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A CYTOLOGICAL STUDY OF ERYSIPHE POLYGONI ON DELPHINIUM [1]

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INTRODUCTION

Recent cytologic and genetic studies of the higher fungi have resulted in a marked alteration of the point of view as to the nature of sex, the process of sexual reproduction, the alternation of generations, and the genetics of fungi. The cytology of the mildews has been much studied in the past, but it has seemed worth while to reexamine the mildews in the light of recent advances in other ascomycetes and in the basidiomycetes.

MATERIAL AND METHODS

Material for this study was obtained from a *Delphinium* plant growing out of doors in a damp, partly shaded place. The plant was growing rapidly and was covered with a luxuriant growth of mildew bearing thousands of perithecia in all stages of development. The material was fixed in Flemming's weak solution and in Flemming's weak solution diluted to two-thirds and to one-half strength, respectively. The fixed material was washed and dehydrated and then embedded in paraffin, sectioned 12μ thick, and stained with Heidenhain's iron haematoxylin with a counter stain of Congo red.

INVESTIGATIONS

MYCELIUM

Plate 1, *A*, represents a detail of the young, superficial, vegetative mycelium of *Erysiphe polygoni* DC. The hyphae consist of long, straight cells, fairly uniform in diameter except at the formation of a haustorium, a vegetative branch, or a conidiophore. No detailed study has been made of the formation of haustoria or the development of the conidiophores.

Vegetative cells of young mycelium are uninucleate. The vegetative nucleus may be located at any point within the cell. It varies greatly in shape (pl. 1, *B, C, D, E, F, G*), but always contains abundant

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chromatin, a large protruding "nucleole" at one end, and usually one or two dark-staining smaller granules located elsewhere on the nuclear membrane.

A comparison of the drawings shown in plate 1, *B* to *G*, suggests that the nucleus possesses the power of locomotion. The shape of the nucleus varies greatly (*B, C, D*) and not infrequently (*E, F, G*) the "nucleole" is prolonged into a slender rod terminating in a small knob which projects out into the cytoplasm. It may be that this nucleolar apparatus is a mechanism for locomotion. Details of the process have not been worked out.

The question arises as to whether a nucleus can pass through a septum from one cell of a hypha to another. Cytoplasmic connection through a septum between adjoining cells is probably always present. These connections are seen most clearly in cells that are slightly plasmolyzed (pl. 1, *H, a*), but in turgid cells also the configuration of the cytoplasm often suggests direct streaming of cytoplasm through the center of a septum. Plate 1, *I*, shows an immature conidium. One both sides of each septum, strands of cytoplasm converge upon the center of the septum. If minute openings in septa are of regular occurrence it would seem possible for a nucleus also to pass through. But on the supposition that nuclei migrate through the mycelium one would expect to find irregularities in the number of nuclei in the cells. A count of 100 vegetative cells gives 97 cells with 1 nucleus and 3 cells with 2 nuclei. These three binucleate cells are easily accounted for as incidental to growth and the formation of new cells. There is no evidence, then, that in purely vegetative mildew mycelium any nucleus leaves the cell of its birth.

DEVELOPMENT OF THE PERITHECIUM

Delphinium mildew has not been proved to be heterothallic, but heterothallism is now known to exist in the sunflower mildew (*59*)[3] and the early stages of reproduction in the two mildews are similar.[4]

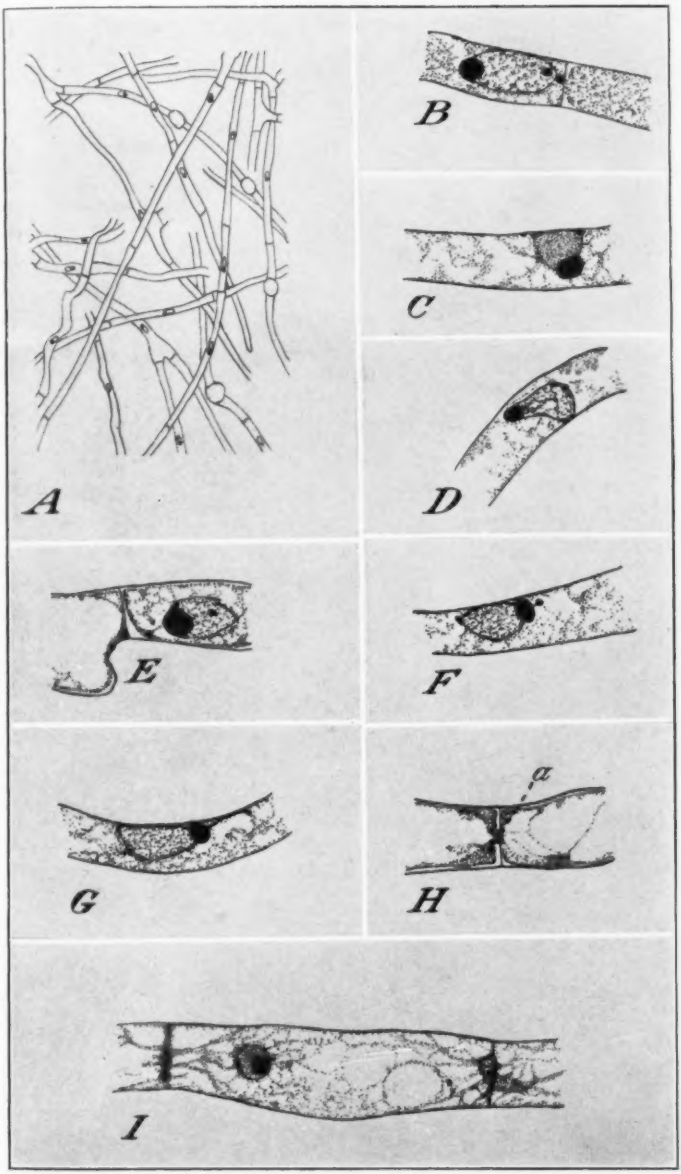
The early stages in the sexual reproduction of delphinium mildew have been studied, and in no case has it been found that the two hyphae concerned originated on the same individual. In sectioned material, of course, the mycelium is cut, and it is rarely possible to trace a hypha far; but, if the species were homothallic, it is unlikely that among the hundreds of cases studied a few would not have been found in which the connection between the two hyphae could be proved. It is probable, although not proved, that there are (+) and (−) strains here, as in sunflower mildew, and that only when the two meet does reproduction take place.

In a mass culture of young vegetative mycelium (supposedly containing both (+) and (−) strains) the hyphae manifest no attrac-

[3] Reference is made by number (italic) to Literature Cited, p. 81[6].
[4] Unpublished data.

EXPLANATORY LEGEND FOR PLATE 1

A.—Young vegetative mycelium. Mass culture. × 230.
B, C, D.—Nuclei of vegetative mycelium showing different shapes and positions. × 2,250.
E, F, G.—Nuclei of vegetative mycelium showing prolongation of nucleole into a slender rod capped by a small knob. × 2,250.
H.—Slightly plasmolyzed mycelial cell showing (at *a*) cytoplasmic connection from one cell to the other through the septum. × 2,250.
I.—Immature conidium showing strands of cytoplasm converging on the center of the septa. × 1,400.



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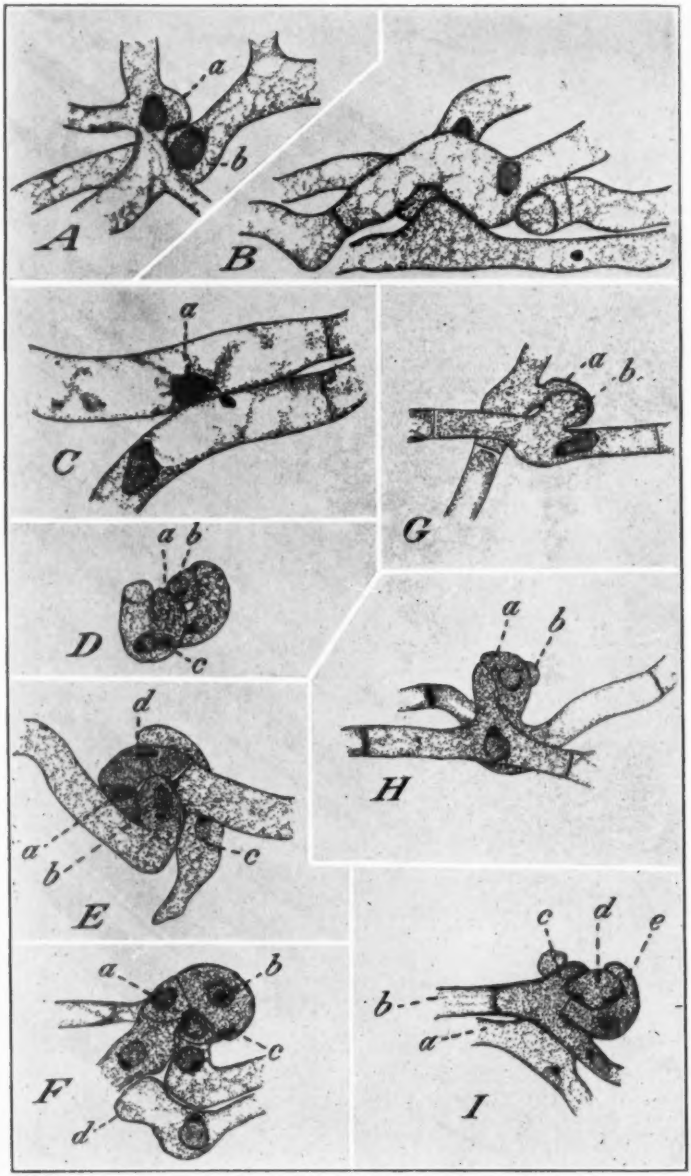

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tion for each other. Contacts between hyphae occur only when the mycelial mat is so dense that contacts are unavoidable. When an accidental contact occurs (pl. 1, *A*) the two hyphae continue their growth with unaltered size and direction.

Quite otherwise is their behavior a day or two later at the inception of reproductive activities. Contiguous hyphae, supposedly (+) and (−), swell toward each other, forming an extensive surface of contact. The first relations between the two present great diversity in appearance. The initial surface of contact between two hyphae may be at the growing tips or farther back, or one growing tip may make contact with an older portion of a second hypha.

Usually only two hyphae are concerned at the start, as in plate 2, *A*. Here one hypha is swelling at *b* and the other is beginning to push out a branch along the line of contact at *a*. Occasionally more than two are involved. Plate 2, *B*, represents an extreme case in which four hyphae have swollen and taken on a sinuous growth, with extensive areas of contact with each other. Rarely the initial contact between two hyphae serves to stimulate the passage of a nucleus from one to the other, as in *C*, *a*.

When the initial contact is between the growing tips of two hyphae (pl. 2, *D*, *a*, *b*), both tips thicken and curl up. The cells are usually uninucleate, the cytoplasm is dense, and dark-staining granules (*D*, *c*), probably food, appear in the cytoplasm. In the further development of this type (*E*) one hypha, *a*, coils around the other, *b*. In this case, a third hypha, *c*, has become associated with them. At *d* is a dark-stained body in the act of passing through a septum. Whether this is a food granule or a much-condensed nucleus is not clear. In plate 2, *F*, the terminal cells, *a*, *b*, of the two hyphae have established communication, and cytoplasm is passing through from *a* to *b* at *c*. Here, also, a third hypha, *d*, is affiliated.

In the majority of cases the initial surface of contact between two hyphae is formed at some distance back from the growing tips. In plate 2, *G*, the two hyphae grew past each other at an angle and thickened at the area of contact, and each is pushing out a short, thick branch. The two young branches (*G*, *a*, *b*) are closely associated and are equal in size and similar in shape. In plate 2, *H*, the branches, *a*, *b*, are larger and somewhat coiled about each other. In this case there is a distinct difference between the two. One, *a*, is shorter, thicker, and less twisted than the other, *b*. In plate 2, *I*, a slightly later stage, the difference is more marked. The hypha *a* has formed a short, thick, curved branch, *d*, and the hypha *b* has produced two slender hyphae, *c* and *e*, which are wrapped around the first. Plate 3, *A*, shows a still later stage. The two hyphae with their branches

EXPLANATORY LEGEND FOR PLATE 2

A.—Beginning of sexual reproduction. Two hyphae in contact, with swellings at *a* and *b*. × 1,400.
B.—Four swollen hyphae in sinuous growth, with extensive areas of contact with each other. × 1,400.
C.—Two mycelial hyphae in contact. A nucleus, *a*, is passing from one hypha to the other. × 2,250.
D.—Area of contact between curled up growing tips of hyphae, *a* and *b*. Granule, possibly food, at *c*. × 1,400.
E.—Growing tips of two hyphae, *a* and *b*, curled about each other. Third hypha at *c*. At *d* a dark-stained body is passing through a septum. × 1,400.
F.—Curved growing tips of two hyphae, *a*, *b*, in communication at *c*. Third associated hypha at *d*, × 1,400.
G.—Two hyphae in contact pushing out branches, *a*, *b*. × 1,400.
H.—Two hyphae with young branches, *a*, *b*, twisted about each other. × 1,400.
I.—Hypha *a* has formed heavy branch, *d*, and hypha *b* has pushed out slender branches *c*, *e*, wrapped about *d*. × 1,400.

are shown in their natural relation in A_1, while in A_2 and A_3 the two are drawn separately. One, A_2, has produced a large, heavy, uninucleate cell with dense cytoplasm, accompanied by a much slenderer branch, while the other A_3, has formed a hypha whose slender branches clasp the large cell of A_2. Two cells in A_3 are binucleate.

In this case (pl. 3, *A*) it would be easy to take for granted that A_2 is the female branch and A_3 the male branch. In some of the other cases presented here, however, it would be difficult to assign sex.

In plate 3, *B*, is shown an irregular case. Three hyphae, *a*, *b*, *c*, are involved. The hypha *af* has formed a thickened branch, which passes under *b* and emerges at *d*. The hypha *b* has formed a large, thick, irregular branch, *e*, which has extensive areas of contact with *c* and with both parts of *a*.

In a few cases the growing tip of one hypha makes contact with an older portion of a second hypha. In plate 3, *C*, a broad surface of contact has formed between the swollen second cell of hypha *a* and the swollen tip of hypha *b* and an opening has formed between the two cells at *c*. Here it would be difficult to decide which is male and which is female. In plate 3, *D*, the tip of hypha *a* met the hypha *bc* at right angles. The latter pushed out a short, thick branch, *e;* the growing tip of hypha *a* thickened and twisted about it; and a broad opening between the two has formed at the apex, *d*. In plate 3, *E*, also, the growing tip of one hypha, *a*, crossed an older hypha, *b*, then stopped growing and formed a thickened binucleate terminal cell, *c*. Meanwhile the older hypha, *b*, thickened and is pushing out slender branches to meet *c*. In plate 3, *F*, the tip of hypha *a* met *b*, then turned and grew parallel to *b*, while slender branches from *b* have grown out to *a* and partly enclosed it.

In the majority of cases the fusion is between terminal cells of reproductive branches. There are exceptions. In plate 2, *C*, already described, a nucleus is passing between two older cells, neither of which is terminal. In plate 3, *C*, also described, the terminal cell of one hypha fused with the second cell of the other. In the example shown in plate 3, *G*, a nucleus is moving from hypha *a* into the third cell of hypha *b*. The nucleole leads in the advance of the nucleus. In plate 3, *H*, the nucleus, *c*, is moving from an intercalary cell of hypha *b* into *a*. As a portion of *a* was removed in sectioning, its position in its own hypha is unknown.

In the further development of the perithecium the narrow initial opening between the two fusing cells seen in plate 2, *C*, *F*, and plate 3, *C*, *D*, *G*, *H*, broadens rapidly. In plate 4, *A*, the fusion between the large cell, *a*, and the smaller cell, *b*, is complete. Two nuclei of unequal size are seen in the large cell (*a*). The fusion cells shown in

EXPLANATORY LEGEND FOR PLATE 3

A.—Two reproductive branches drawn together in their natural relation in A_1 and drawn separately in A_2 and A_3.

B.—Hypha *af* formed heavy branch passing under *b* and emerging at *d*. Hypha *b* formed heavy branch, *e*, which has broad areas of contact with *af*, *ad*, and *c*.

C.—Enlarged tip of hypha *b* in communication at *c* with enlarged second cell of hypha *a*.

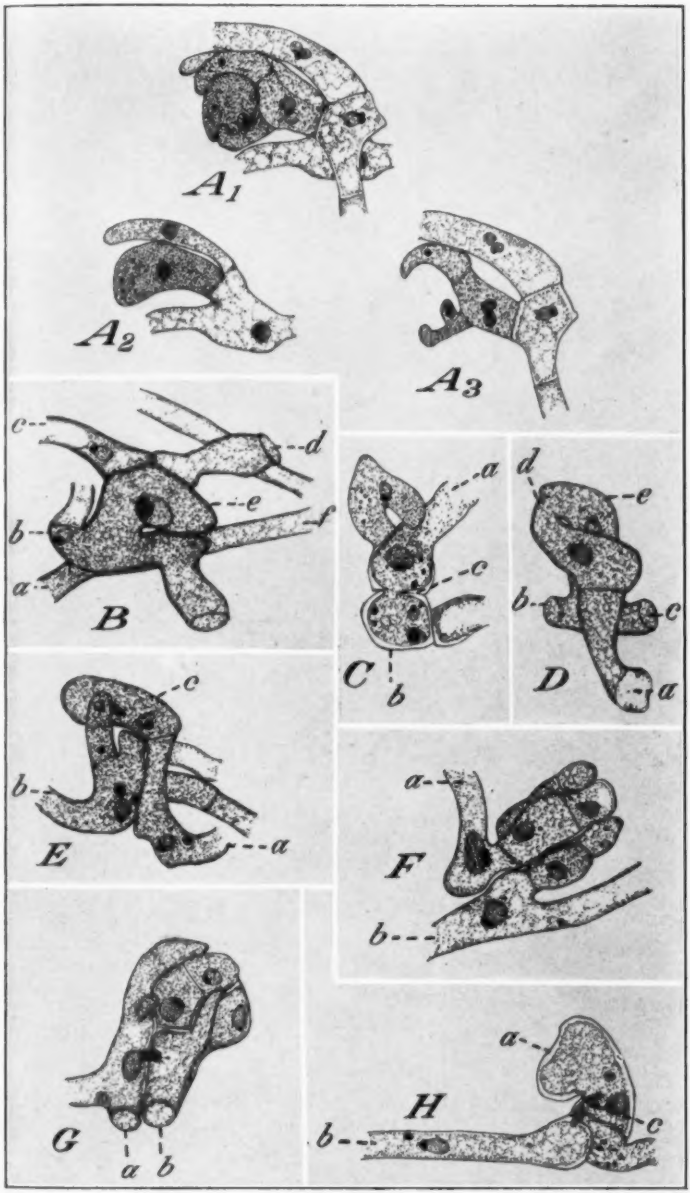
D.—Hypha *bc* formed heavy branch *e*, which is in communication at *d* with enlarged tip of hypha *a*.

E.—Binucleate terminal cell, *c*, of hypha *a*, stopped growing and thickened. Slender branches from hypha *b* are pushing out to meet *c*.

F.—Hypha *a* met *b*, then turned and grew parallel to it. Hypha *b* is pushing out branches to *a*.

G.—Nucleus from hypha *a* moving into third cell of hypha *b*.

H.—Nucleus, *c*, from hypha *b* moving into hypha *a*. All × 1,400.



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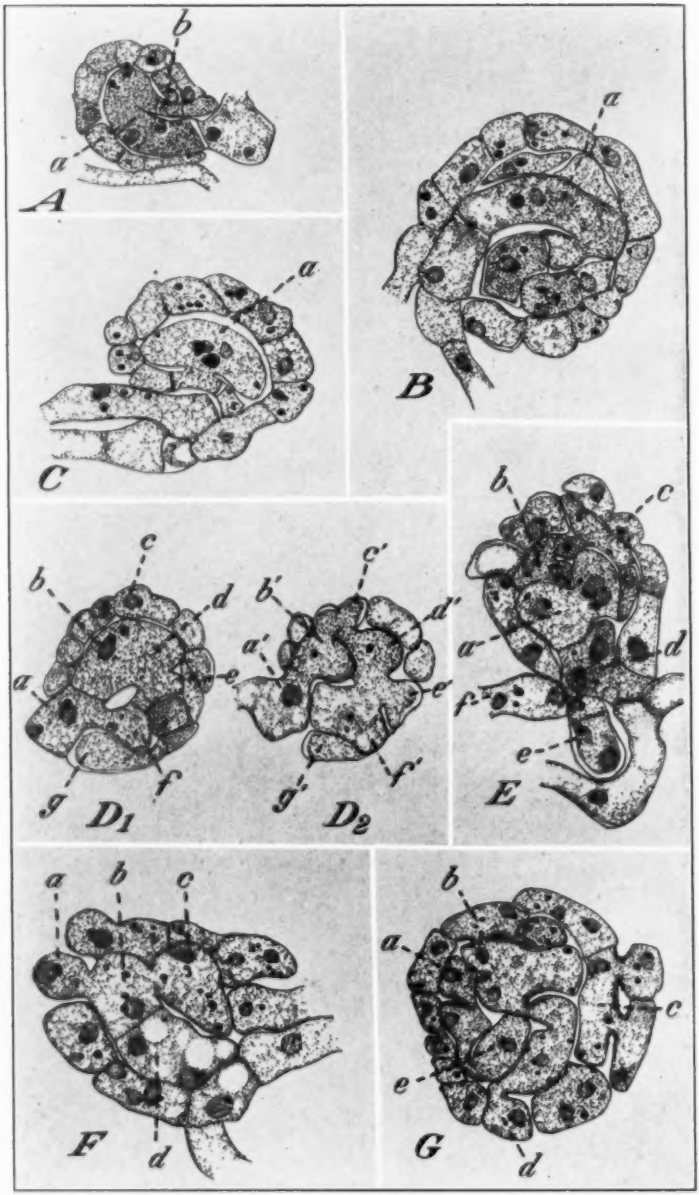

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plate 4, *B* and *C*, are similar except that in *C* there are three nuclei in the center at *a*. No indication of a nuclear fusion at this time has been seen. During this time a layer of enveloping hyphae (the beginning of which has been seen in pl. 3, *A* and *F*) has formed, enclosing the central cell.

At first sight, plate 4, *A*, *B*, *C*, would seem to be clear evidence that two well-defined cells, an antheridium and an oogonium, fuse, and that their nuclei become associated. But drawing a perithecium of this age at a carefully chosen focus, as was done in *A*, *B*, *C*, can give a false impression of the situation.

By focusing up and down through a section (cut 12μ thick), it can be determined that many of the cells of these little perithecia are in open communication with one another. Plate 4, D_1 and D_2, illustrate this. The plane represented in D_1 is similar to that in *A* and *C*, but by focusing down, the picture changes gradually to D_2. Careful focusing up and down shows that cell *a* is continuous with *a'*, *b* with *b'*, *c* with *c'*, etc., and the fact emerges that a large portion of this little perithecium, including at least a part of its peripheral layer, forms a continuous passageway, and that the combination is multinucleate. A similar study of the perithecia drawn in *A*, *B*, *C* shows that in them also there are continuous mazes of communicating chambers.

As the point is of theoretic interest, it was studied further. In plate 4, *E*, the large trinucleate central cell, *a*, consists of two nearly equal lobes. Note the branching multinucleate maze, *b*, and the successive constrictions in *c*. The stalk cells, *d*, *e*, and *f*, are binucleate.

In the great majority of perithecia of this age it is still possible to locate the initial point of fusion. In the perithecium drawn in plate 4, *F*, however, it is uncertain whether the initial fusion took place in the three-lobed series *a*, *b*, *c*, or in the binucleate central cell, *d*. Perhaps even here the fusion could have been located with certainty if the perithecium had been cut at some other angle.

In plate 4, *G*, is drawn a transverse section through a perithecium. At the right, an added peripheral layer of the perithecium is forming by the proliferation of the cell *c*. A continuous passageway can be traced through the whole diameter of this perithecium from *b* to *d*. Another shorter maze runs from *a* to *e*. The question arises as to how much of one of these mazes should be considered one cell. As mentioned earlier (p. 802), it is probable that in vegetative mycelium minute openings in the septum between one cell and the next are regularly present. It may be that in young perithecia these openings between cells are much larger and more conspicuous. If this is true the segment of a maze between each constriction and the next would be one cell.

EXPLANATORY LEGEND FOR PLATE 4

A.—Young perithecium. Cells, *a* and *b*, fully fused.
B.—Fusion cell, *a*, with two nuclei.
C.—Fusion cell, *a*, with three nuclei.
D.—D_1 and D_2 show two different levels in the same section of a perithecium, in which *a* is continuous with *a'*, *b* with *b'*, etc.
E.—Perithecium with trinucleate central cell, *a*, short mazes at *b* and *c*, and binucleate stalk cells, *d*, *e*, *f*.
F.—Perithecium with short maze, *a*, *b*, *c*, and binucleate cell *d*.
G.—Transverse section of perithecium with maze from *b* to *d*, and another from *a* to *e*. New outer layer of perithecium forming from *c*. All × 1,400.

In plate 5, *A*, is shown a longitudinal section of a perithecium with its mycelial connections. Several hyphae contributed to the formation of this perithecium. The maze of communicating cells is so intricate and complete that one gains the impression that more than one fusion was necessary to produce it. In cases like this it would be impossible to determine how much of such a maze formed as a result of growth and cell divisions with incomplete closure of the septa, and how much, if any, arose through subsidiary fusions.

When two cells and the opening between them lie in one horizontal plane, the fact is easily determined. When a cell connects with another above or below it, it is much more difficult to be sure. It is theoretically possible that, in some cases at least, if all the connections in a small perithecium could be determined the whole perithecium would be found to consist of one intricate communicating system. This has not been proved. Moreover, the extent of the intercommunication seems to vary in different perithecia. The perithecia in plate 5, *B* and *C*, for example, show the minimum of connections. A few connections are to be seen here, and others occur in these same perithecia in planes above and below the one drawn; but it is highly improbable, in these perithecia at least, that all the cells are connected.

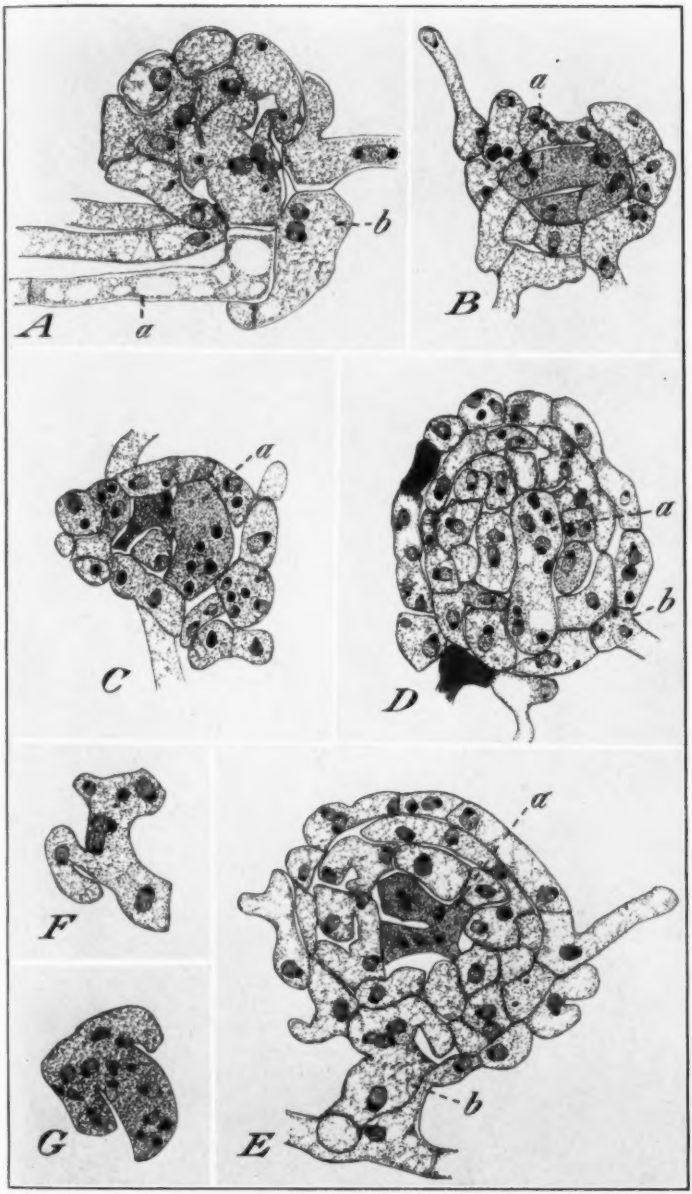
During this development there is a rapid increase in the number of nuclei. The increase is most conspicuous in the fusion cell. Directly after fusion the central cell contains two or, at most, three nuclei (pl. 4, *A*, *a*; *B*, *a*; *C*, *a*; *E*, *a*). Soon after this it contains four or five (pl. 5, *B*, *a*), then six or eight (pl. 5, *C*, *a*; *D*, *a*; *E*, *a*; *F*), and still later a dozen or more (pl. 5, *G*).

It is not the fusion cell only that shows this increase in the number of nuclei. A survey of plates 4 and 5 shows that throughout the young perithecium the number of nuclei is on the increase. The question arises as to the source of these nuclei. Do they form by nuclear divisions in the perithecium or do nuclei migrate in from the mycelium? The nuclear content of stalk cells connecting the perithecium to the mycelium is most variable. In plate 5, *A*, the stalk cell at *a* contains no nuclei; the one at *b* contains two. The mycelial connection at plate 5, *D*, *b*, contains three nuclei, and the one at plate 5, *E*, *b*, contains four. Four and five nuclei are common in the stalk cells in plate 6, *B*, and plate 8, *C*.

If surplus nuclei are forming in the mycelium and moving into the perithecium, the nuclear content of mycelial cells would be irregular. As stated earlier, a count of 100 cells in young vegetative mycelium shows that 97 are uninucleate and 3 are binucleate. A similar count in mycelium bearing numerous small perithecia shows that of 100 mycelial cells 88 are uninucleate and 12 binucleate. There may be a slight justification here for the supposition that extra nuclei form in the mycelium and migrate to the perithecia, but if this occurs it is probably a minor factor. Moreover, only once or twice in this older

EXPLANATORY LEGEND FOR PLATE 5

A.—Longitudinal section of perithecium showing mazes. Mycelial stalk cell at *a* with no nucleus and one at *b* with two nuclei.
B and *C*.—Perithecia with few short mazes. Multinucleate central cell at *a*.
D.—Larger perithecium with multinucleate central cell, *a*, and trinucleate stalk cell, *b*.
E.—Perithecium with multinucleate decadent central cell, *a*, and multinucleate stalk cell, *b*.
F and *G*.—Multinucleate central cells. All × 1,400.



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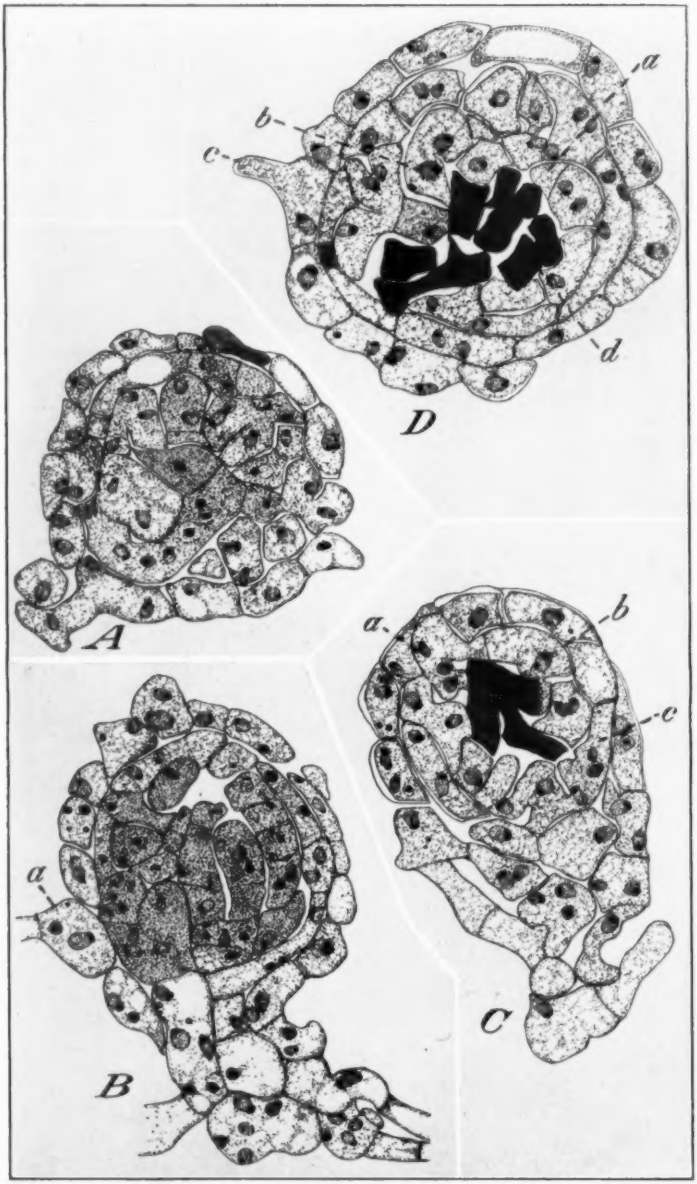

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mycelium has a nucleus been found in the act of passing through a septum from one mycelial cell to another.

An attempt has been made to determine the nuclear content of perithecial cells. Such an attempt must, of course, result in an underestimate of the number of nuclei per cell, for in sectioned material many cells are cut in two, and some of their nuclei appear in one section and others in another. No attempt was made to piece the two together. For the purpose of these counts, each segment of a maze was considered a cell. The results of the counts are given in table 1.

TABLE 1.—*Number of nuclei in cells of perithecia of different ages and in different parts of perithecia*

PERITHECIA OF DIFFERENT AGES

Young perithecia (4 cells or less in diameter)			Slightly older perithecia (5 to 7 cells in diameter)		
Nuclei per cell (number)	Cells		Nuclei per cell (number)	Cells	
	Number	*Percent*		*Number*	*Percent*
1	60	60	1	131	65.5
2	33	33	2	59	29.5
3 or more	7	7	3 or more	10	5
Total	100	100	Total	[1] 200	100

DIFFERENT PARTS OF PERITHECIA [1]

Two outer layers of perithecium			Central area of perithecium		
Nuclei per cell (number)	Cells		Nuclei per cell (number)	Cells	
	Number	*Percent*		*Number*	*Percent*
1	106	69	1	25	55
2	45	29	2	14	30
3 or more	3	2	3 or more	7	15
Total	154	100	Total	46	100

[1] The same 200 cells of slightly older perithecia have been regrouped according to their location in the perithecium.

As may be seen from the data in table 1, approximately one-third of the cells in young perithecia carry more than one nucleus, and the percentage of cells with more than one nucleus in the outer layers of a perithecium is nearly as large as in the central area. Of course, if a count could be made in unsectioned perithecia, the proportion of cells with more than one nucleus would be found to be greater than one-third. If a whole maze were to be considered as one cell, nearly all (perhaps all) the cells would be counted as multinucleate.

In summing up the observations on the early stages of development of the perithecium, the conclusion seems justified that following the fusion of the two initial cells growth takes place with incomplete closure of the septa, and that through the mazes thus established nuclei (presumably both (+) and (−)) become widely distributed,

EXPLANATORY LEGEND FOR PLATE 6

A and *B*.—Older perithecia with high nuclear content and all cells living. Young appendage at *B*, *a*.
C.—Longitudinal section of perithecium with dead multinucleate central cell, *b*, and mazes at *a* and *c*.
D.—Transverse section of perithecium with group of dead cells at *d*, cells starting to grow centripetally at *a* and *b*, and young appendage at *c*. All × 1,400.

thus diploidizing a large part of both the outer and inner layers of the perithecium.

During the next stages of growth the oldest cell in the perithecium (i. e., the cell formed by the fusion of the first two cells at the initiation of the perithecium) dies. The time at which the death of the central cell occurs varies somewhat. In plate 6, *A* and *B*, all cells are still living, while in the younger perithecia shown in plate 5, *C* and *E*, the central cell is beginning to die. In the latter (pl. 5, *C*, *a*, and *E*, *a*) the cytoplasm of the central cell stains darkly and the nuclei are beginning to shrink. The numerous nuclei left in the central cell at this time do not leave it but die with it. In the perithecium drawn in plate 6, *C*, the central cell, *b*, is dead, but its dozen or more nuclei can still be seen there shrunken and dead.

Commonly a little later a few other cells most closely associated with the central cell also die. This is shown in plate 6, *D*, *d*.

When young perithecia with dying central cells were first encountered, it was taken for granted that they were, for some unknown reason, decadent and degenerating. When this condition was found repeatedly, such an explanation appeared more and more doubtful. It was remembered, also, that the mildew used for this study grew out of doors under conditions ideal for its development and that the mildew was making a most luxuriant growth.

In order to learn how general this death of the oldest, first-formed cells of the perithecium was, a survey was made of over 100 perithecia. The data on these were first classified according to the size of the perithecium. Perithecia five cells in diameter were placed in one group, those six cells in diameter in another, etc. Then the perithecia within each size group were classified according to whether all cells were living, only the central cell was dead, or two to six cells of the central area were dead. The results are shown in table 2.

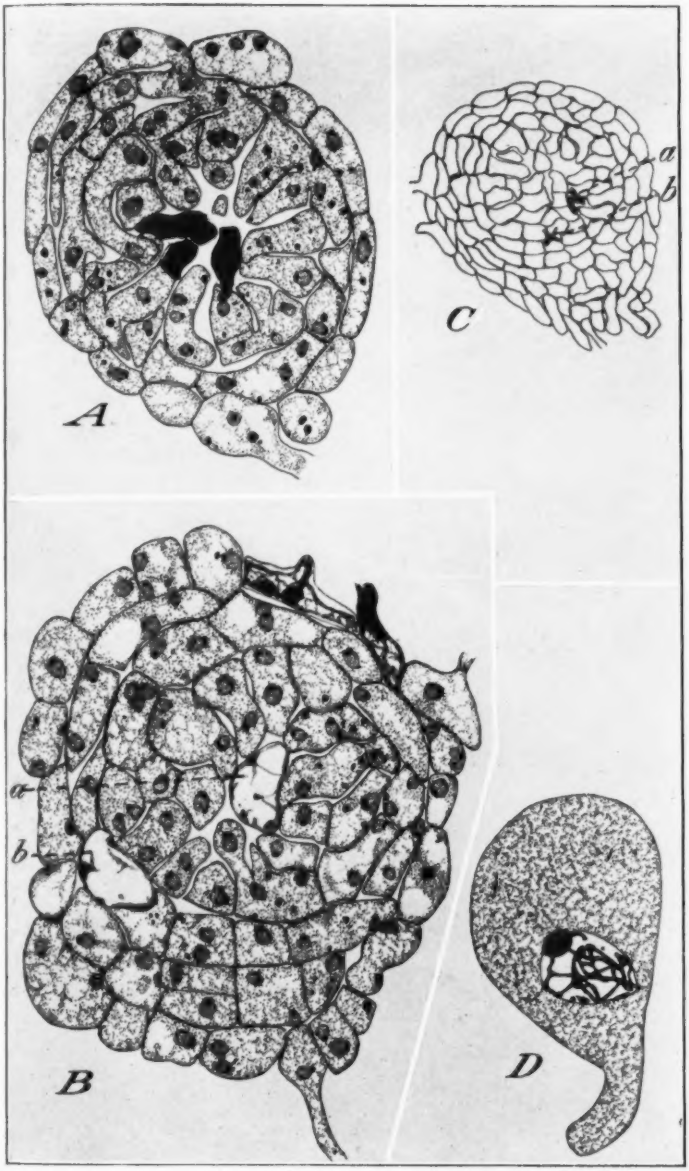
TABLE 2.—*Classification of young perithecia according to size and to the presence of dead cells within them*

Size of perithecia (number of cells across diameter)	Perithecia with—			Total perithecia
	All cells living	Central cell dead	Two to six cells dead	
	Number	*Number*	*Number*	*Number*
5	30	8	0	38
6	12	7	0	19
7	8	8	3	19
8	6	7	7	20
9	0	4	3	7
10	2	1	7	10
11	0	0	2	2
Total	58	35	22	115

From table 2 it can be seen that in perithecia five and six cells in diameter the majority show all cells living. In about one-fourth,

EXPLANATORY LEGEND FOR PLATE 7

A.—Perithecium with shrinking dead cells in center, surrounded by layer of centripetally growing cells. × 1,400.
B.—Perithecium with centripetal layer, dead cells at *a* and *b*, and base becoming heavier. × 1,400.
C.—Diagram of older perithecium. Remnant of dead cells at *a*. Asci will soon form at *b*. × 500.
D.—Ascus showing prophase of heterotypic division. × 1,400.



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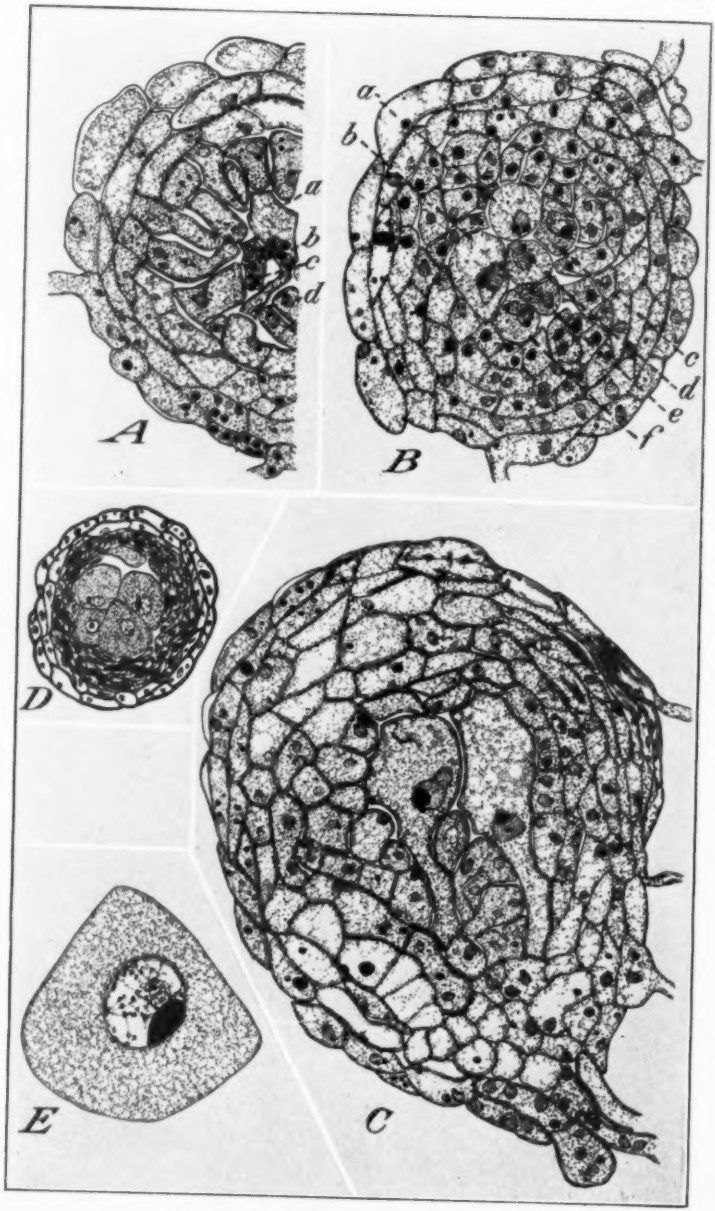

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the initial fusion cell is dead. In perithecia of the seven-cell size group and larger, the number of perithecia with all cells living rapidly decreases and the number of those with more than one cell dead increases. It is exceptional to find a perithecium more than eight cells in diameter with all cells living.

This death of the oldest, first-formed cells of the perithecium does not hinder the later development. Long before these cells die many of the progeny of the two (rarely three) nuclei present in the fusion cell at the beginning have become broadly distributed throughout the growing perithecium. In the perithecium drawn in plate 6, *C*, the central cell, *b*, is dead, but the vigorous multinucleate mazes at *a* and *c* carry on the growth.

As the perithecium continues to grow, the two outer layers become differentiated as wall layers. These cells grow and divide to keep pace with the expanding perithecium. Certain of the surface cells in the equatorial region of the perithecium or slightly below it push out to form the appendages (pl. 6 *B*, *a*; *D*, *c*).

Just inside the wall of the perithecium a layer of cells grows inward, usurping the space and perhaps resorbing the substance of the dead cells. A beginning of this is seen in plate 6, *D*, where cells at *a* and *b* are turning inward. This centripetal growth is much more pronounced in the perithecium shown in plate 7, *A*, where cells all around the circle are pointed inward and the dead cells that they center upon are much shrunken.

Plate 7, *B*, shows a later stage. Although the central space is filled in solid, the layer of inward-growing cells can still be seen, while at *a* and *b* are two separated dead cells, pale and nearly empty but not collapsed. The base of the perithecium is becoming heavier as tangential divisions increase the number of cell layers.

So far as noted, in all later cell divisions in the perithecium septa are completed. No longer are openings left between daughter cells. The mazes have disappeared, suggesting that earlier openings are now closed.

Plate 7, *C*, drawn at lower magnification than *A* and *B*, shows diagrammatically the condition of the perithecium just before the formation of asci. The structure has become more massive. The central area of centripetal growth is still discernible. Between it and the surface there are now several cell layers, of which the outer layers will become the wall. Small remnants of the dead cells are seen at *a*. The asci will spring from the region indicated by *b*.

Plate 8, *A*, shows part of a section through a perithecium in which asci are beginning to form. An ascus starting at *d* forced its way upward to *a* against the pressure of the surrounding cells. It is irregular in form, being indented by each cell that it encountered in its growth. Its two nuclei at *b* are already larger than the nuclei

EXPLANATORY LEGEND FOR PLATE 8

A.—Half a section of a perithecium showing young ascus, *ad*, its two nuclei at *b*, and a remnant of a dead cell at *c*. × 1,020.

B.—Transverse section of perithecium showing young binucleate asci, *e*, *f*, nuclei fusing in asci at *a* and *d*, and asci with nuclear fusion nearly completed at *b* and *c*. × 1,020.

C.—Longitudinal section of perithecium showing two larger uninucleate asci in center and multinucleate stalk cells below. × 1,020.

D.—Diagram of transverse section of older perithecium, with four large uninucleate asci in center. Surrounding cells crushed and dying. × 320.

E.—Lower right ascus of *D*, enlarged. Its nucleus is in prophase of heterotypic division. × 1,400.

of nearby vegetative cells. At *c* is the shrunken remnant of one of the dead cells.

In a longitudinal section through a perithecium, two or at most three of the asci are seen. A median, transverse section cuts across all the asci. In the center of the cross section drawn in plate 8, *B*, is a nest of six asci cut at the level that shows their nuclei. The asci at *e* and *f* are the youngest and smallest of the group and are still binucleate. In the asci at *a* and *d*, nuclear fusion is in progress. In each, the two nuclei have joined but the nucleoles are still some distance apart. In the asci at *b* and *c* the nucleoles also have fused. Finer details of the nuclear fusion have not been worked out.

After the nuclear fusion the ascus enters upon a prolonged period of growth, during which it remains uninucleate. In plate 8, *C*, is shown a longitudinal section through a larger perithecium showing two uninucleate asci in the central area. In plate 8, *D*, is drawn at low magnification a transverse section through a much older perithecium. The asci have grown rapidly at the expense of the surrounding cells, which are now shrunken and dying. The lower right-hand ascus of this perithecium is drawn enlarged in plate 8, *E*. The outline is plump, the cytoplasm dense, and the large nucleus is in a prophase of the heterotypic division. In plate 7, *D*, is shown a longitudinal view of a uninucleate ascus during the later part of this growth period.

The later development within the ascus has not been studied.

DISCUSSION

The mycelium of powdery mildew is superficial, and under natural conditions is exposed to the weather. New cells formed during growth of hyphae harden quickly. When sexual reproduction begins, hyphae (supposedly (+) and (−)) are attracted to each other, as is evidenced by the formation of extensive areas of contact with each other. Rarely a nucleus moves through directly from one hypha to the other at the time and place of the first contact. It is surprising that a nucleus could effect a passage necessary for this transfer through the two older hardened walls.

The powdery mildew of sunflower (*Erysiphe cichoracearum* DC.) grows readily on separate sunflower leaves floated on sugar solution in covered Petri dishes and forms mature perithecia there with normal spores. Here the mycelium is growing indoors in diffuse light and in very humid air. At the beginning of sexual reproduction, under these protected conditions,[5] the hyphae show the same attraction for each other and form the same areas of contact as in delphinium mildew, but here the passage of a nucleus directly from one older hypha to another is fairly common. It is natural to assume that when grown in a damp chamber in this way, the cell walls of the hyphae remain thinner or softer and offer less obstruction to the passage of a nucleus than when grown out of doors. This mode of fertilization is achieved without the formation of any reproductive branches. There is nothing here that could be labeled an "antheridial" or "oogonial" branch.

[5] Unpublished data.

Occasionally the growing tips of two ordinary mycelial hyphae of delphinium mildew meet, stop growing in length, swell, curl, and an opening forms between the tips, thus initiating a perithecium. Here, also, fertilization takes place without the formation of special reproductive branches, for the joined apices of two ordinary hyphae serve as the point of communication. It may be pointed out that the apex of any hypha would have a thin, soft wall that would facilitate the formation of such an opening.

Not uncommonly the pair of cells that fuse consist of the growing tip of an ordinary mycelial hypha and a short side branch from an older part of a second hypha. One gets the impression from studying such cases that as the tip of one hypha grows past the other an attraction between the two occurs that inhibits the apex of the one from growing farther and incites the formation of one or more side branches on the other. Here, one of the two cells that fuse is a special branch formed in connection with reproduction.

Commonly, and in perhaps the majority of cases, the attraction and first contact are between mature hard-walled parts of two adjoining hyphae and each of the two pushes out a short side branch, thus bringing together young growing tips from the two.

In other words, the essential fact of sexual reproduction here is the meeting of two hyphae (perhaps (+) and (−)), an opening between the two, and a nuclear transfer that brings into one cell two different nuclei. It would appear to depend on circumstances whether the transfer is (1) between older cells of two adjoining hyphae; (2) between the growing tips of two ordinary hyphae; (3) between one growing tip and a short side branch from an older hypha; or (4) between two short side branches, one from each of two older juxtaposed hyphae. Given attraction and contact between two hyphae, the nuclear transfer will take place, either directly, if the walls are soft enough to permit it, or after the formation of one or of two small side branches. Even when the two short side branches are formed, there is great variety in their shape, size, and relationship, and while it is usually the terminal cells of the two that fuse, it is not necessarily so. Moreover, it is not uncommon to find one of the two cells binucleate before fusion.

According to published accounts of the Erysiphaceae (*5*, *7*, *8*, *43*, *44*, *46*, *49*), the perithecium is regularly and uniformly initiated by the formation on adjoining hyphae of two characteristic short side branches, one bearing the antheridium, the other the oogonium. These specialized sex cells become slightly twisted on each other, an opening forms between the two at the apex, and a nucleus passes through from the antheridium to the oogonium.

Figures resembling the published drawings of antheridia and oogonia in powdery mildew are easily found in *Erysiphe polygoni*, but along with these there are so many other types as to suggest that, in this species at least, the formation of special sex cells is a matter of expediency rather than a necessity.

In a broad range of ascomycetes the occurrence of well-defined antheridia and oogonia has been recorded (*4*, *5*, *11*, *12*, *13*, *20*, *31*, *40*, *41*, *42*, *44*, *45*, *47*, *53*, *57*). They differ in form in different species but are fairly uniform for a given species. The following exceptions and irregularities have been noted:

In *Lachnea stercorea* Pers., Fraser (*33*) finds that both oogonia and antheridia form but no antheridial nuclei enter the oogone. The ascogone, none the less, develops, and a normal ascocarp forms. Winge (*58*) finds the same condition in *Sphaerotheca*. Dangeard (*15, 16, 17, 18*) takes the extreme stand that, while antheridia and oogonia are of general occurrence in ascomycetes, it is in only a few of the most primitive that one or more nuclei pass from the antheridium to the oogonium. In all others no nuclear transfer occurs.

In some species (*1, 2, 3, 21, 26, 28, 29, 30, 35, 36, 60*) all individuals produce oogonia but no antheridia. Notwithstanding this, the ascogonia develop normally. Recent work shows that in some of these species every individual produces oogonia and also produces some form of conidia, oidia, microconidia, or spermatia, which replace antheridia in function. Heterothallism has been proved in several of these species. Only when (+) conidia are brought into contact with a (−) individual (or (−) conidia with a (+) individual) do fertilization and development of the ascocarp take place.

The mode of fertilization in these cases varies greatly in different species. In *Sclerotinia gladioli* Massey (*28, 29, 30*) all individuals produce microconidia and columnar receptive bodies. The microconidia cannot grow into mycelium and can function only when microconidia from a (+) individual are placed on the specialized receptive bodies of a (−) individual, or vice versa. In heterothallic strains of *Neurospora* (*21*), on the contrary, either the conidia or the microconidia of one sex can effect fertilization of an individual of the other sex, either directly or after growing into mycelium. Two microconidia, one (+) and one (−), can grow into mycelia that mate with each other and produce normal perithecia. Moreover, in recent work Dodge (*24, p. 425*) has proved that in *Neurospora tetrasperma* (normally bisexual and only facultatively heterothallic), "a bisexual mycelium can be obtained as the result of a vegetative union or anastomosing of hyphae from two unisexual mycelia of opposite sex reaction."

In *Bombardia lunata* Zickler (*60*) every individual, (+) or (−), forms both ascogones and spermogonia. A spermogonium is a single flask-shaped cell within which a dozen or more minute uninucleate spermatia are formed. This species also is heterothallic.

In *Humaria granulata* Quel. (*9*) every individual forms oogonia but no antheridia. Later work (*37, 38, 39*) shows that the species is heterothallic, for a single-spore culture remains sterile, but there are neither antheridia nor any form of conidia. Mating is achieved by mycelial anastomoses between (+) and (−) individuals, which lead to the development of normal fruits.

In *Penicillium wortmanni* Klöcker, a homothallic species, Emmons (*32, p. 133*) finds that "a cell or slightly differentiated branch of the vegetative mycelium functions as an ascogonium, and no paired organs are to be found."

In *Humaria rutilans* Fries neither antheridia nor oogonia are formed. A knot of thickened hyphae develops and "fusions of nuclei in pairs occur in the hypothecium, constituting a process of reduced fertilization or apogamy" (*34*).

In these few examples there are all gradations from the typical sexual reproduction by means of oogonia and either antheridia or some form of microconidia to a type with complete absence of formal

sex cells. Lohwag (*51*) has brought together and discussed a wide range of material showing still further variations.

The present studies of *Erysiphe polygoni* show that even a species that regularly produces antheridia and oogonia can show other, less formal, types of reproduction, including even occasional instances of mycelial mating.

It was earlier believed that the passage of a nucleus (or of more than one when antheridia are multinucleate) from the antheridium to the oogonium was immediately followed by nuclear fusion (*8*, *11*, *12*, *43*, *44*, *45*, *46*).

The puzzling fact was often discussed, however, that there is also a nuclear fusion in the ascus. The existence of two nuclear fusions in one life cycle demanded an explanation. In 1907 Claussen (*13*) discovered that in *Pyronema confluens* Tul. the male nuclei entering the oogone pair with the female nuclei but do not fuse with them, that this pairing is followed by the formation of ascogenous hyphae with paired nuclei, and that these give rise to binucleate asci in which, finally, nuclear fusion takes place.

Although some writers since then still believe in the existence of nuclear fusions in both the oogone and the ascus (*31*, *40*, *41*, *42*, *47*, *55*, *56*), the majority believe that in the ascomycetes, as in the basidiomycetes, the initial cell fusion is not accompanied by nuclear fusion but leads into a more or less prolonged binucleate phase of development (with conjugate divisions of paired nuclei), terminated in the ascus or basidium by nuclear fusion. Swingle (*55*) has tabulated the results of nearly 60 pieces of research on this subject. In *Erysiphe polygoni* no evidence has been seen of nuclear fusion at the time of cell fusion.

In the typical ascomycete as recorded in the literature the fertilized oogone or ascogone is soon sheathed by sterile gametophytic hyphae that grow up from below. Their further growth provides the sterile portion of the fruit body. At the same time the ascogone itself gives rise to a special set of ascogenous hyphae with paired nuclei which in turn give rise to the asci.

Erysiphe polygoni differs markedly from this. Fertilization is followed by growth in which the septa remain incomplete for a considerable period, so that a large number (perhaps sometimes all) of the cells of the perithecium are in open communication with one another for a time, and many cells in all parts of the perithecium contain more than one nucleus. There is no definite limited set of ascogenous hyphae embedded in sterile growth, for much of the perithecium, including even a part of its peripheral layer, is sporophytic in character.

No reference has been found in the literature to a similar case in the ascomycetes. The nearest approach is *Neurospora tetrasperma*. Colson (*14*) finds that there are no well-defined ascogenous hyphae in the young perithecium. There is a central "fertile area" composed of multinucleate cells. Here (*14*, *p. 214*) "cross walls are rare and difficult to see." From this area the asci arise.

Hints of a similar condition in other ascomycetes are found in the literature. Of *Humaria granulata*, Gwynne-Vaughan says (*38*, *p. 142*): "The whole ascocarp, in fact, is derived from the union of (+) and (−) mycelia, and is formed of dithallic cells." Harper (*43*), Winge (*58*), and Homma (*49*) have figured cells with more than one nucleus in vegetative parts of the perithecium of different species of *Sphaerotheca*, but no significance was attached to this condition. In species of

ascomycetes with several-cell ascogones the septa between the cells may be incomplete. This was found by Harper (*44*) in *Ascobolus furfuracens* Pers., by Fraser (*35*) in *Lachnea cretea* Phil., by Schweizer (*52*) in *Ascobolus citrinus* Schw., and by Drayton (*29, 30*) in *Sclerotinia gladioli*. In these cases there was no mention of the formation of mazes in the later growth. In the beginning the ascogenous hyphae in many ascomycetes, however, are nonseptate and multinucleate. It seems probable that other ascomycetes will be found whose young fruit body is made up largely of communicating sporophytic cells derived directly from, or at least connected with, the fertilized ascogone.

In *Erysiphe polygoni* the ascogone and a few of the cells most closely associated with it usually die when the perithecium is seven or eight cells in diameter. This probably is common in ascomycetes. Harper (*44*) notes its occurrence in *Erysiphe communis* (Wallr.) Fr. and *Ascobolus furfuracens*. Blackman and Fraser (*9*) describe the same condition in *Humaria granulata*.

After the death of the ascogone and associated cells in *Erysiphe polygoni*, cells (usually multinucleate) of the inner layer of the perithecium wall grow centripetally. Centripetal growth also has been seen by Harper (*44*) in *Erysiphe* and *Ascobolus*.

Knowledge of heterothallism and homothallism in the ascomycetes is still limited. Table 3 gives a list of species (probably incomplete) that have been proved to be heterothallic.

TABLE 3.—*Species that have been proved to be heterothallic*

Species	Year	Author	Reference to literature cited
Ascobolus carbonarius Karst.	1926	Betts	(*6*)
Ascobolus magnificus Dodge	1920	Dodge	(*20*)
Ascobolus stercorarius (Bull.) Schröt.	1931	Dowding	(*26*)
Bombardia lunata Zickler	1934	Zickler	(*60*)
Ceratostomella pluriannulata Hedgcock	1932	Gregor	(*36*)
Diaporthe perniciosa Marchal	1931	Cayley	(*10*)
Gelasinospora tetrasperma [1]	1933	Dowding	(*27*)
Humaria granulata Quel.	1930	Gwynne-Vaughan	(*38*)
	1927	Shear and Dodge	(*54*)
Neurospora [1] (several species)	1932	Dodge	(*21, 22, 23*)
	1932		
	1934		
Pleurage anserina (Ces.) Kuntze	1931	Dowding	(*25*)
Sclerotinia gladioli Massey	1932	Drayton	(*28, 29, 30*)
	1934		

[1] *Gelasinospora tetrasperma* (*27*) and *Neurospora tetrasperma* (*21*) are normally bisexual and homothallic. The ascospores are binucleate. Occasional dwarf ascospores are uninucleate and these grow into unisexual mycelium. These are heterothallic. A mating of 2 such unisexual mycelia of opposite sex is necessary for the production of perithecia.

There are other anomalous cases difficult to classify. Hüttig (*50*) found in *Glomerella lycopersici* Krüger a homothallic type which in single-spore culture produced perithecia freely, also a second type which in single-spore culture produced only small brown sclerotium-like bodies, but when mated with the first type, behaved as male. When exposed to heat, cold, or roentgen rays, still other types appeared with differing sex capabilities which "erfüllt die Forderung der Theorie der relativen Sexualität von Hartmann."

Homothallism frequently has been reported in ascomycetes. In earlier work, however, evidence was accepted as proof of homothal-

lism which since has been found to be inadequate. The absence of antheridia does not prove homothallism, for antheridia may be replaced functionally by microconidia, or even by mycelial anastomoses between individuals of opposite sex. The formation of antheridium and oogonium as branches of the same hypha does not prove homothallism, for their development may have been preceded by mycelial fusions with nuclear migrations. Even growing a single-spore culture which forms normal ascocarps does not prove homothallism unless special precautions are taken, for Dowding (*26*) has shown that mites carrying oidia can crawl between a Petri dish and its cover and so serve as agents for fertilizing cultures in closed Petri dishes. The list of reported cases of homothallism in ascomycetes would shrink considerably if judged by the present critical standards.

Evidence of heterothallism also is being scrutinized more critically. Derx (*19*) earlier reported *Penicillium luteum* (Zukal) Wehmer to be heterothallic. Recent work by Emmons (*32*) calls this in question, for "Our single spore cultures have produced abundant ascospores." Species of *Penicillium* often "become nonascosporic in culture", and Derx may have encountered a strain which in a single-spore culture failed to produce ascospores, not because it was a haploid but because of a loss of fertility in culture.

Until recently no tests for heterothallism or homothallism had been reported for the Erysiphaceae. Now, Yarwood (*59*) has proved the powdery mildew of sunflower to be heterothallic, and Homma (*48*, *49*) reports that *Sphaerotheca fuliginea* (Schlecht.) Poll. is homothallic. Yarwood is now conducting the experimental work on this point in *Erysiphe polygoni*.

SUMMARY

In vegetative mycelium of *Erysiphe polygoni* DC., cells are long, straight, and uninucleate. Hyphae show no attraction for each other. There is some evidence of cytoplasmic streaming through septa.

In sexual reproduction hyphae swell toward each other, forming broad areas of contact. A nucleus may pass directly from one hypha to the other, or communication may be established at the joined apices of two hyphae or between short side branches on adjoining hyphae.

The opening between the two fusing cells broadens rapidly. No indication has been seen that nuclear fusion occurs in the fusion cell.

Cell fusion is followed by growth with incomplete closure of the septa, resulting in the formation of chains of communicating cells or mazes, which make up the bulk of all parts of the perithecium, including even its peripheral layer. These cells, evidently sporophytic in character, contain from one to several nuclei. In the later growth the septa of the mazes are completed and further cell divisions reduce the number of nuclei per cell.

When the young perithecium is seven or eight cells in diameter, the initial fusion cell and usually a few of the cells most closely associated with it die. As the perithecium expands cells of the inner wall of the perithecium grow centripetally, filling the central space. From the lower central area five or six cells grow up to form the asci. Each ascus contains at first two nuclei, which soon fuse.

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HYBRID VIGOR AND GROWTH RATES IN A MAIZE CROSS AND ITS RECIPROCAL[1]

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INTRODUCTION

With the development and rapid commercial utilization of hybrid corn, the phenomenon of hybrid vigor is becoming of increasing practical importance. The theoretical aspects of hybrid vigor have received considerable attention, but certain phases of the phenomenon have not yet been adequately explained. The two current theories as to the cause of hybrid vigor are (1) physiologic stimulation due to the interaction of unlike germ plasms and (2) dominant favorable growth factors. Observational genetic evidence and results from convergent improvement studies (*12*)[2] are in agreement with the second hypothesis.

Ashby (*1*, *2*) has reported on a series of experiments which he interprets as requiring some modification of the dominant-favorable-growth-factor hypothesis and which seem not to be in accord with extensive observations on hybrid vigor in maize.

He (*1*, *p. 458*) suggests that hybrid vigor may be manifested in four ways:

> 1. More meristematic centres may be present in the hybrid, giving more leaves and tillers. 2. The leaves of the hybrid may be more efficient in photosynthesis. 3. The embryo may be bigger, so that the capital with which the hybrid germinates is bigger. 4. Finally, the falling off in the sigmoid curve of growth might occur much later for the hybrid than for its parent * * * [and concludes that] Hybrid vigour in these strains is nothing more than the maintenance of an initial advantage in embryo size.

This conclusion assumes that reciprocal hybrids differing in embryo weights must also differ in the weight of the mature plant.

Extensive observations of reciprocal maize hybrids have indicated that such hybrids are essentially similar in their general plant development unless distinct differences in seed condition are involved. Since the phenomenon of hybrid vigor in maize is of such great practical as well as theoretical importance, and since Ashby's conclusions are so contrary to the beliefs commonly held by corn breeders, it was decided to repeat Ashby's experiments in considerable detail.

MATERIAL AND METHODS

Later generations of the inbred strains of corn (*Zea mays* L.) used by Ashby (*2*), 228–4–8, 228–6–5, and their reciprocal hybrids, were employed in this study. In 1933 the material was grown at the Arlington Experiment Farm, Arlington, Va., near Washington, D. C.,

[1] Received for publication June 16, 1936; issued January 1937. The data on which this paper is based were obtained as a part of the cooperative program conducted by the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Field Crops Department of the Missouri Agricultural Experiment Station.

[2] Reference is made by number (italic) to Literature Cited, p. 829.

and in 1935 at Columbia, Mo. The two seasons were very dissimilar, 1933 being quite favorable for corn and 1935 very unfavorable.

The method of planting was the same in each year, the plants being checked in hills 3.3 feet apart in each direction. The two inbred lines were compared directly, one plant of each line being grown in each hill. The same system was used for the two reciprocal hybrids. Plantings made in this manner had two advantages when inbred was compared with inbred or hybrid with hybrid: (1) Sampling errors were reduced because of the elimination of correlated variation, and (2) competition should accentuate any differences in growth rate which might exist. These advantages do not apply to the important comparison of inbred with hybrid. It is possible that this planting arrangement may actually have reduced the reliability of such comparisons.

Ten plants of each inbred and hybrid were harvested at 5-day intervals throughout the growing season. All harvests were made from perfect-stand hills, hills adjacent to a missing hill being discarded. In harvests made after silking, plant and ear weights were recorded separately. The data presented deal almost exclusively with plant weights.

A comparison of growth rates for the 1933 crop was made by two methods. The first consisted in fitting a straight line to the dry weights obtained from the first nine harvests. A measure of the significance of slope is provided by Fisher (*5*). The second method consisted in fitting a curved regression line of the form $Y=a+bx+cx^2+dx^3$ . . . to the entire period of growth. The y values were the differences between the logarithms of the dry weights at each harvest period. According to Fisher's terminology, a' is the mean difference and b' and c' are proportional to the difference in slope and change in slope, respectively. The contributions of these various items (a', b', c', etc.) to the total variance can be readily measured.

EXPERIMENTAL RESULTS

1933 CROP

The dry weights of seeds, embryos, and successive plant harvests for the inbred parents and their reciprocal hybrids are available. The weights of shelled grain at successive harvests were obtained in only one season and then only for the hybrids; as these data are somewhat unrelated to the rest of the material to be presented, they will be considered first. The detailed results are presented in table 1. The yields are below normal and somewhat erratic. This may be a result of flooding; water stood 6 feet deep over these plots between August 22 and 24, at which time the ears were in the milk stage. Regardless of the variability, the grain yields of the reciprocals are not significantly different in trend or at the final harvest period.

TABLE 1.—*Dry weight of shelled grain of the hybrid 4–8×6–5 and its reciprocal at successive harvests in 1933*

Date of sampling	Weight [1] of shelled grain per plant		Date of sampling	Weight [1] of shelled grain per plant	
	4-8×6-5	6-5×4-8		4-8×6-5	6-5×4-8
	Grams	*Grams*		*Grams*	*Grams*
Aug. 7	26.1±1.27	27.5±2.97	Sept. 2	165.8±5.57	158.4± 7.12
Aug. 12	49.4±3.67	41.6±3.51	Sept. 6	149.6±7.28	146.4±11.63
Aug. 18	110.0±4.71	91.2±7.08	Sept. 11	149.4±8.81	144.4± 7.35
Aug. 29	149.1±5.60	133.8±5.39	Sept. 16	173.0±4.62	162.6± 6.12

[1] The standard error, and not the probable error, is shown.

The dry weights for seeds, embryos, and plants of the successive harvests are presented in table 2. Here, as in Ashby's results, the inbred 228–6–5 has the smaller kernel and embryo. The difference in embryo weights for the two inbreds is not significant, whereas the difference between the reciprocal hybrids is significant. The logarithms of the weights of the plants at the successive harvests are shown graphically in figures 1 and 2.

TABLE 2.—*Dry weights of seeds, embryos, and plants at successive harvests of two inbred lines of corn and their reciprocal hybrids in 1933*

Part of plant or date of harvest	Individuals in sample	Mean dry weight[1] for indicated parent or hybrid			
		4–8 selfed	6–5 selfed	4–8×6–5	6–5×4–8
	Number	*Grams*	*Grams*	*Grams*	*Grams*
Seeds	50	0.2228±0.0018	0.1891± 0.0021	0.2575±0.0012	0.2314±0.0023
Embryos	50	.0029± .0005	.0026± .0004	.0035± .0006	.0020± .0007
Plants harvested:					
May 29	10	.060 ± .012	.041 ± .004	.105 ± .002	.078 ± .005
June 3	10	.129 ± .023	.088 ± .010	.270 ± .022	.197 ± .006
June 8	10	.388 ± .042	.289 ± .010	.812 ± .059	.626 ± .031
June 13	10	1.643 ± .226	.785 ± .096	3.926 ± .206	2.511 ± .341
June 18	10	2.862 ± .430	1.369 ± .084	8.309 ± .267	6.535 ± .448
June 23	10	6.909 ± .956	2.853 ± .221	18.476 ±1.40	13.286 ±1.25
June 28	10	15.31 ± .856	10.74 ± .646	31.399 ±2.90	25.008 ± .993
July 3	10	27.51 ±2.50	18.05 ± 2.06	62.8 ±5.13	60.6 ±5.50
July 8	10	34.29 ±2.83	31.35 ± 1.36	93.0 ±5.18	86.6 ±6.28
July 13	10	44.77 ±4.61	35.32 ± 2.94	136.1 ±3.96	124.7 ±4.86
July 18	10	63.80 ±5.47	53.0 ± 3.57	167.8 ±7.70	175.2 ±4.33
July 23	10	93.25 ±5.92	63.25 ± 5.81	204.1 ±7.81	190.4 ±4.53
July 28	10	97.00 ±6.52	94.38 ±12.27	233.6 ±6.48	234.3 ±3.70
Aug. 2	10	147.25 ±5.83	115.25 ± 5.56	246.0 ±8.10	240.6 ±8.25
Aug. 7	10	175.50 ±6.78	122.75 ± 5.47	275.6 ±6.45	279.7 ±6.56

[1] The standard error, and not the probable error, is shown.

The straight-line regressions, fitted to the logarithms of the dry-weight determinations for the first 45 days for the two parental lines and their reciprocal hybrids, were as follows:

Parent or hybrid:	*Value of y*
228–4–8	$0.07277+2.53064x$
228–6–5	$.07390+2.29566x$
228–4–8 × 6–5	$.07579+1.81698x$
228–6–5 × 4–8	$.07858+1.64024x$

The differences in the slope of the four regression lines as indicated by the regression coefficients are not statistically significant. This suggests that during the 45-day period the inbreds and their reciprocal hybrids all grew at approximately the same rate.

An analysis of the data for the entire growth period by means of the curved regression line leads to essentially the same conclusion. The F values (*15*) for the various constants are presented in table 3. In no case is there a significant difference in slope (b'). This indicates no significant dissimilarity of growth rates during the period studied.

The difference between the mean dry weights (a') is significant in every comparison. For the reciprocal hybrids, however, the difference in mean dry weight is of limited biological significance, as the two hybrids do not differ statistically in weight after the 40-day period. In the comparisons of the change in the slope of the regression lines (c'), the inbred lines in each case differ significantly from the hybrids because of the earlier cessation of growth in the hybrid (fig. 1). With the exception of the failure to find a continuing difference between reciprocal hybrids, the results are in apparent harmony with those presented by Ashby.

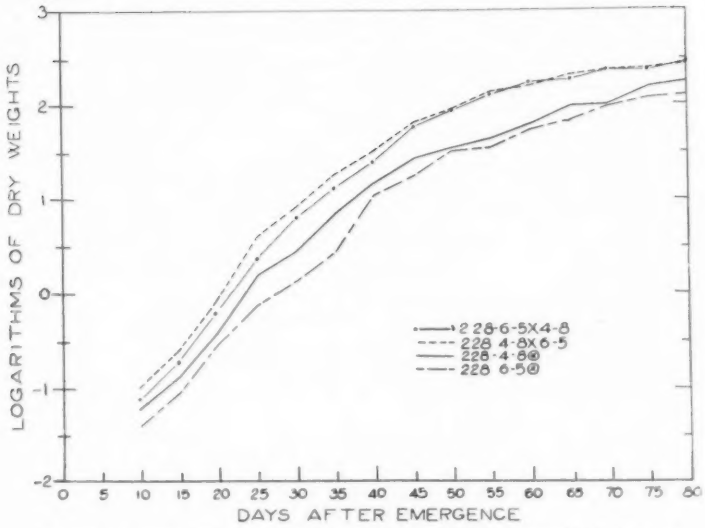

FIGURE 1.—Growth rates of two inbred strains of corn and their reciprocal hybrids in 1933.

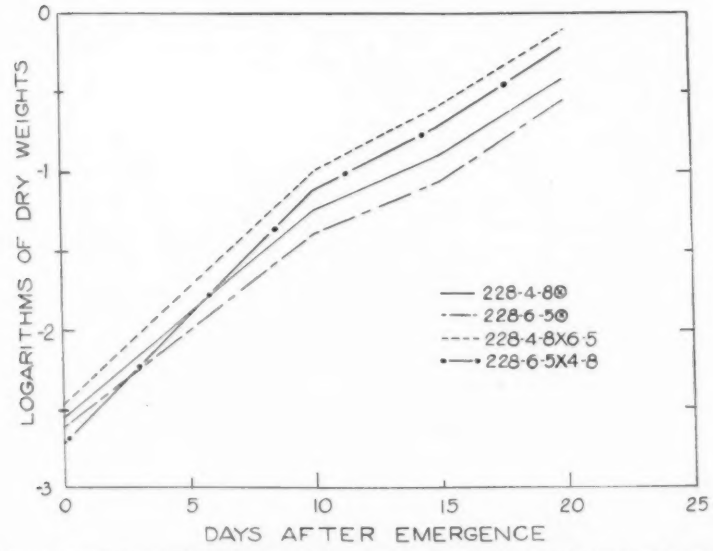

FIGURE 2.—Growth rates of two inbred strains of corn and their reciprocal hybrids during the early seedling stage in 1933.

TABLE 3.—*Comparisons of the regression constants of two inbred lines of corn and their reciprocal hybrids grown in 1933*

Comparison	Value [1] of F for indicated constant		
	a'	b'	c'
4–8 selfed and 6–5 selfed	50.68	0.40	0.43
4–8 selfed and (4–8 × 6–5)	562.16	.04	19.94
6–5 selfed and (6–5 × 4–8)	274.35	.00	11.35
(4–8 × 6–5) and (6–5 × 4–8)	18.18	1.00	.22

[1] 4.84 is the lowest value F may take for significance when $P=0.05$; 9.65 is the lowest value F may take for significance when $P=0.01$.

Before proceeding further with the analysis it is desirable to evaluate the reasonableness of the indicated conclusions. If the dry weight at the end of the 45-day period is expressed as a percentage of the initial weight, the values for the inbreds 4–8 and 6–5 and the hybrids 4–8 × 6–5 and 6–5 × 4–8 are 572, 765, 886, and 1,110, respectively. When each hybrid is compared with its maternal inbred, the ratio of these percentages is approximately 1.5 to 1. This indicates that the hybrid actually has grown at a rate approximately 50 percent greater than its maternal inbred parent. The failure to establish the significance of this difference may be due to several causes. The sampling may have been inadequate to represent the population, or the variability from sample to sample so great that a very large difference in growth rate would be necessary for significance.

On the basis of the variability observed in the nine harvests covering the initial 45-day period, a difference in growth rate of 75 percent would be necessary to give odds of 20 to 1. As indicated previously, a difference of approximately 50 percent was observed. The difference necessary for significance covering the entire growth period has not been calculated but it doubtless would be somewhat greater than for the 45-day period.

The results published by Ashby appear less conclusive when one considers the percentage increase in dry matter as a measure of growth rate. Expressing the final harvest weight as a percentage of the initial harvest weight and comparing the faster growing inbred with its F_1 hybrid, differences of 21 and 46.9 percent are found for the material presented in his first and second study, respectively. In each case he concluded that the inbred and hybrid were equivalent in growth rate. It is clearly evident that the high degree of variability present in Ashby's and in the writer's material precludes any very definite conclusions as to the equivalence of growth rate in inbreds and hybrids.

1935 CROP

The detailed data for the 1935 harvests are presented in table 4 and are shown graphically in figure 3. In contrast to the 1933 data, the inbred 228–6–5 has the higher embryo and grain weight. The hybrid 6–5 × 4–8 has a smaller grain and embryo weight than the maternal inbred itself. The reciprocal hybrid, however, shows an increase in both grain and embryo weight over its maternal inbred parent. These variations in embryo weight are probably the result of variation in soil fertility and relative plant development. Selfed

seed produced on a favored inbred plant may have larger kernels and embryos than hybrid seed produced on a less favored plant of the same inbred strain.

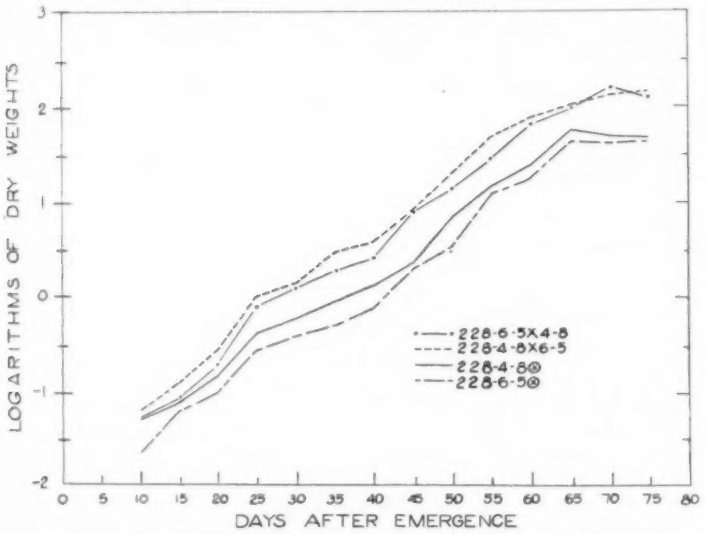

FIGURE 3.—Growth rates of two inbred strains of corn and their reciprocal hybrids in 1935.

TABLE 4.—*Dry weights of seeds, embryos, and plants at successive harvests of two inbred lines of corn and their reciprocal hybrids in 1935*

Part of plant or date of harvest	Mean dry weight [1] for indicated parent or hybrid			
	4–8 selfed	6–5 selfed	4–8 × 6–5	6–5 × 4–8
	Grams	*Grams*	*Grams*	*Grams*
Seeds	0.215 ±0.007	0.265 ±0.001	0.246 ±0.002	0.225 ±0.001
Embryos	.0031± .0003	.0034± .0005	.0035± .0003	.0029± .0002
Plants harvested:				
June 18	.053 ± .003	.024 ± .001	.066 ± .003	.054 ± .002
June 23	.096 ± .007	.060 ± .004	.136 ± .005	.086 ± .003
June 28	.159 ± .039	.104 ± .011	.284 ± .020	.201 ± .013
July 3	.434 ± .015	.302 ± .006	1.027 ± .030	.825 ± .079
July 8	.578 ± .025	.427 ± .012	1.401 ± .110	1.317 ± .034
July 13	.902 ± .074	.538 ± .009	3.191 ± .248	2.086 ± .191
July 18	1.307 ± .116	.719 ± .039	3.786 ± .217	2.612 ± .189
July 23	2.433 ± .287	2.179 ± .294	8.444 ± .562	8.111 ± .959
July 28	7.110 ± .413	3.571 ± .448	21.700 ±1.277	14.500 ± .633
Aug. 2	15.69 ± .563	12.679 ± .464	47.662 ±3.543	37.297 ±2.602
Aug. 7	25.116 ±1.902	18.900 ±1.489	77.116 ±1.704	72.137 ±5.304
Aug. 12	58.548 ±1.277	44.506 ±2.075	105.971 ±2.546	99.930 ±3.405
Aug. 17	48.848 ±1.410	45.415 ±1.033	132.619 ±2.049	153.614 ±8.702
Aug. 22	50.482 ±4.549	45.512 ±4.593	141.288 ±6.345	128.460 ±8.434

[1] The standard error, and not the probable error, is shown.

The difference in weight of embryo for the hybrid 4–8 × 6–5 and its reciprocal was not very great in either year. In order to obtain a greater difference in embryo weight, one set of the hybrid ears 6–5 × 4–8 was harvested April 20 and another set was harvested

May 4. This material was grown in the greenhouse and the sequence of silk and pollen prevented making the cross reciprocally. The difference between the embryo weights of the prematurely and normally harvested corn was slightly greater than that of the two lots of reciprocal hybrids previously compared. This initial difference in embryo weight, however, was ineffective in modifying the expression of vigor. The mean dry weights of plants (table 5) from the two lots of seed are quite similar for practically every harvest. There is no significant difference between the final dry weights.

TABLE 5.—*Mean dry weights of seeds, embryos, and plants at successive harvests of the hybrid (6–5 × 4–8) harvested before and at maturity in 1935*

Part of plant or date of harvest	Mean dry weight [1] for the hybrid 6–5 × 4–8 at—		Part of plant or date of harvest	Mean dry weight [1] for the hybrid 6 5 × 4–8 at—	
	Premature harvest [2]	Normal harvest		Premature harvest [2]	Normal harvest
	Grams	*Grams*		*Grams*	*Grams*
Seeds	0.214 ±0.001	0.278 ±0.002	Plants harvested—Contd.		
Embryos	.0027± .002	.0036± .0002	July 23	13.111±0.926	14.777±1.445
Plants harvested:			July 28	33.300±1.352	43.300±2.553
June 18	.054 ± .002	.065 ± .003	Aug. 2	76.319±3.600	80.734±1.910
June 23	.112 ± .006	.124 ± .008	Aug. 7	107.064±1.524	113.034±4.068
June 28	.288 ± .017	.342 ± .022	Aug. 12	130.846±2.170	167.182±6.135
July 3	.933 ± .056	.883 ± .035	Aug. 17	145.350±3.116	180.586±7.259
July 8	1.719 ± .059	1.891 ± .058	Aug. 22	169.565±6.384	163.583±6.426
July 13	3.757 ± .277	3.848 ± .274			
July 18	5.138 ± .468	5.353 ± .807			

[1] The standard error, and not the probable error, is shown.
[2] The premature harvest was 2 weeks earlier than the normal harvest.

The evaluation of the curved regression constants is presented in table 6. The difference between mean dry weights (a') is significant in every comparison. However, as pointed out for the 1933 results, the difference between the reciprocal hybrids is of little practical importance. The final plant weights were not significantly different. The difference in rate of growth (b') for the two inbreds probably is significant, the value for P lying between 0.01 and 0.05. The difference in growth rate between the 228–4–8 inbred and the hybrid 4–8 × 6–5 is clearly significant. The inbred 228–6–5 and the hybrid 6–5 × 4–8 do not differ significantly in slope, though the F value approaches the 5-percent point. The ratio of the percentage growth for this hybrid and its maternal inbred for the initial 45-day period is 1.8 to 1.0, or an indicated difference in growth rate of 80 percent.

TABLE 6.—*Comparisons of the regression constants of two inbred lines of corn and their reciprocal hybrids grown in 1935*

Comparison	Value of F_1 for indicated constant		
	a'	b'	c'
4–8 selfed and 6–5 selfed	41.272	5.491	0.183
4–8 selfed and (4–8 × 6–5)	387.172	15.953	21.018
6–5 selfed and (6–5 × 4–8)	279.818	4.249	6.375
(4–8 × 6–5) and (6–5 × 4–8)	19.324	3.901	1.505
(6–5 × 4–8) from prematurely and (6–5 × 4–8) normally harvested seed	16.211	.028	.144

[1] 4.96 lowest value F may take for significance when $P=0.05$. 10.04 lowest value F may take for significance when $P=0.01$.

DISCUSSION

In a recent study Engledow and Pal (*4*) concluded that Ashby's explanation of hybrid vigor in maize was inapplicable to wheat. The following discussion is concerned with the agreement and discrepancies of the data presented here and the expectations based on Ashby's conclusions. The discussion will be facilitated by considering each of his postulates separately. His first postulate is that the F_1 hybrid of a cross between two inbreds exhibits the growth rate of the faster growing parent. In a consideration of this postulate it is desirable to make some subdivision of the growth period. The following subdivision is in harmony with the sampling periods and appears useful: (1) From fertilization to maturation of the seed, (2) from germination through the early seedling stage, and (3) from the late seedling stage to mature plant. The first two stages are essentially similar and are subdivided only because they are separated in time by the dormancy of the mature embryo. It should be emphasized that Ashby's studies were concerned almost entirely with the third stage of growth.

Inbred and hybrid embryos can be produced on the same ear and must therefore have their origin in egg cells of the same average weight. As any conceivable difference in weight of sperms from the two types of pollen could not cause more than a trivial difference in weight of the zygote, the inbred and hybrid zygotes are necessarily of similar weight. Since hybrid embryos in general are heavier than selfed embryos,[3] it is clear that the logarithmic growth lines must differ in their slope in the first period. A large amount of data relating to the mature weights of selfed and hybrid seeds has been presented by Kiesselbach (*8*).

Information regarding the second phase of growth is very meager. However, on the basis of the data illustrated in figure 2, it appears that during germination and for at least the first 15 days of seedling growth the rates for inbred and hybrid are decidedly different.

The extensive data dealing with the third stage of growth cannot be used as a critical test of this postulate. Under conditions in which sampling errors obscure differences in growth rates of the magnitude of 50 percent any conclusions as to the equivalence of growth rate in inbred and hybrid are unwarranted.

Lindstrom (*10*) has presented data which he interprets as a disproof of Ashby's hypothesis. He removed a portion of the leaf area of the hybrids at different stages and compared their final growth with that of the inbred parents. The F_1 hybrids exceeded the inbred parents in final plant weight, and he concludes that "This can only mean that the cut-back F_1 hybrids grew at a faster rate than their untouched parents." The writer is in agreement with the idea that F_1 hybrids grow at a faster rate than their parental inbreds. There is some doubt, however, that this has been conclusively proved by the above experiments. The clipping of leaves does not remove "capital" in the sense in which Ashby and physiologists in general have used the word. The tissues removed are no longer meristmatic but are fully differentiated and have attained approximately their ultimate size. If one considers the clipping to have affected only the "net rate of interest" or the "efficiency index" then the results are not necessarily at variance with Ashby's conclusions. The reduction in leaf area and conse-

[3] This statement applies only to inbred or closely line-bred material.

quently the reduction in efficiency index would be expected to be temporary. Even under conditions in which the hybrid and inbred grow at exactly the same rate, the hybrid starting with the larger capital could suffer a temporary reduction in efficiency index and still produce a greater final weight than the inbred parents. But, assuming that the tissues removed by clipping of leaves do represent capital, data showing that the dry matter of the decapitated hybrid was reduced to equivalence with the parental inbred would be essential before one could consider a difference in growth rate as having been proved.

Although the present data are in many respects inadequate, they do indicate that for the first two phases of growth the rate for the hybrid is in excess of that for either parent. It has not been possible to demonstrate a statistically significant difference in growth rate of hybrid and inbred during the third period, but it has been demonstrated that only very large differences would appear significant because of the large sampling errors involved. The data suggest that the difference in growth rates between inbred and hybrid may not be as great during this stage as in the two earlier stages. A possible explanation for this may be in the type of growth involved. Within 25 to 35 days after planting, the morphological foundation of the corn plant is largely completed; the growing point becomes differentiated into a tassel, and growth by cell division becomes of minor importance. Growth by cell differentiation and enlargement has been increasing in importance during the period and from this point on becomes the dominating element.

The presence of two distinct phases of growth in the life of an organism appears to be a common phenomenon in biology. Huxley (*7, p. 118*) in this connection states:

> In the first, the general form of the part is being laid down, and this process is accompanied by very rapid alterations of form, and by marked histological changes; in the second, histological changes are absent or of an entirely secondary nature, and the form changes are confined to quantitative alterations in the proportion of the definitive structural plan. * * * Thus not only definitive form-plans, but also marked differences in size, are established in the short first phase, and effects of growth during the second phase are confined to a quantitative modification of the already diversified organization given at the close of the first phase.

Similar growth rates during a particular stage of development have been reported for markedly differing size structures in a wide variety of organisms. The works of Houghtaling (*6*) on tomatoes, of Sinnott and Kaiser (*14*) on *Cucurbita*, and of Dobzhansky and Duncan (*3*) on *Drosophila* larvae are only a few of the many cases that might be cited.

Ashby's second postulate, namely, that rate of growth is inherited as a simple dominant or dominant complex contributed by one of the parents, does not appear to be true for either the first or second growth period. Because of the large sampling errors, the data presented here on the third growth period cannot be considered as critical. However, every person who has had experience with corn breeding can cite numerous instances of the expression in the hybrid of characters from the two parents that certainly are related to growth rate, as, for example, leafiness and the possession of a good root system.

Ashby's third postulate, namely, that reciprocal hybrids differing in embryo weight should differ in degree of vigor, is only partially

substantiated. The initial difference between such hybrids persists for a time but disappears before the completion of growth. The results for both seasons are in agreement on this point. It seemed desirable to compare the growth rate of hybrids in which greater differences in embryo weights were involved. These differences were obtained by comparing normal and prematurely harvested seed. The data from this source are presented in tables 5 and 6. Here again initial differences in embryo size failed to persist. It has not been possible to show a difference in growth rate in such cases, but it seems probable that the explanation for the failure of such differences to persist lies in the slightly longer growth period of the hybrid having the smaller embryo. In every case such a hybrid comes into sexual maturity slightly later than its reciprocal having the larger embryo. These results are in essential agreement with those presented by Passmore (*11*) on reciprocal *Cucurbita* hybrids.

In the present experiments, variations in embryo size within a genotype have not been of importance. This is in agreement with the work of Kotowski (*9*), on peas, beans, and cabbage; Passmore (*11*), on *Cucurbita*; and Robinson (*13*) and others, on corn.

Ashby's fourth postulate, namely, that hybrid vigor is nothing more than the maintenance of an initial difference in embryo size, is not supported by these results. In both 1933 and 1935 the hybrid 6–5 × 4–8 actually had a smaller embryo than the inbred 228–6–5. As this hybrid and its maternal inbred have been shown to have the same efficiency index within the limits of sampling error, this postulate would require that the hybrid be inferior to or at least no better than the inbred. Actually the hybrid weighed more than twice as much as the inbred parent at maturity. The increased size of the hybrid is in part accounted for by a more rapid growth rate during the second growth phase.

Ashby was of the opinion that his results required some modification of the dominant-favorable-growth-factor hypothesis. This does not appear to be essential. His results could be interpreted quite simply on the assumption that the vigor of the hybrid is due to the inheritance of a large embryo size from one parent and a rapid growth rate from the other. Each of these components is the resultant of the action of a large number of factors. The dominant-favorable-growth-factor hypothesis does not require that the hybrid exceed the parents in every respect, but rather that the combined effect of all factors shall produce a greater end product. The most favorable factors contributed by each parent find expression in the hybrid. This interpretation is in accord with the fact that favorable genic modifications tend to be dominant.

The results of these experiments are of some interest in connection with the two theories on the nature of hybrid vigor. The differing growth rates during the various growth stages find a ready explanation on the basis of the dominant-favorable-gene hypothesis, in the assumption that genes have a specific time of action. The more rapid growth rate of the hybrids during the first two stages of growth would thus be attributed to the complementary action of genes which produce their characteristic effect early in the development of the organism.

The results are more difficult to explain on the theory that hybrid vigor is due to a physiologic stimulation resulting from unlike germ

plasms. If the data presented can be considered as typical, then one must assume that stimulation increases from the first to the second growth period and decreases with the third.

The mechanism by which hybrid vigor is expressed is still unknown, but, as the difference in growth rate between hybrid and inbred is greatest during the period when size increase is primarily by cell division, it is suggested that rate of cell division must be one important factor. Further studies on this problem are under way.

SUMMARY

Data are presented on the growth rates of two inbred strains of corn and their reciprocal hybrids.

The growth period from fertilization to maturity of the corn plant is divided into three phases: (1) From fertilization to maturity of seed, (2) from germination through the early seedling stage, and (3) from the late seedling stage to maturity.

By means of this classification it is shown that the hybrids grew faster than either parent during the first two phases of growth, but differences in the rate of growth during the third period have not been established statistically because of large sampling errors. The differences in the amount of growth are quite significant.

In the first two phases, the inheritance of growth rate cannot be ascribed to a complex from one parent only. The rate must represent the action, complementary or additive, of factors from both parents. The data presented are not critical for the third period.

The reciprocal hybrids were alike in growth rates and in total amounts of growth. Within a genotype, embryo weights were found to be of little importance in determining final weight, as initial differences in embryo weight failed to persist.

Hybrid vigor cannot be attributed to the maintenance of an initial difference in embryo size.

The differing growth rates for the three phases of growth are readily explained on the basis of the complementary action of dominant genes.

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THE OPTICAL ROTATORY POWER OF EXTRACTS OF DERRIS AND CUBE ROOTS[1]

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INTRODUCTION

Several years ago the author (*8*)[2] stated that the rotenone content of derris and cube roots could not be determined by measuring the optical rotatory power of their extracts. Other components of unknown optical activity are present, and hence the values obtained differ widely from the actual rotenone content determined by extraction. The possibility remained, however, that such a determination might give an indication of the insecticidal value of these materials. The appearance of recent articles on this subject has stimulated interest in this possibility. Danckwortt, Budde, and Baumgarten (*3*), for example, have proposed a method of determining toxicity of derris roots that is based on the optical rotatory power of their benzene extracts. Rowaan (*12*) criticized this method, stating that dextrorotatory materials are present and interfere with the results. Danckwortt (*2*) replied that values obtained by optical rotation correspond with the effectiveness of the root. Fischer and Nitsche (*5*) have recently stated that a polarimetric method gave the best indication of the insecticidal effect of a number of derris and rotenone preparations against certain insects.

Some time ago the author, in cooperation with other members of the Bureau of Entomology and Plant Quarantine (*9*), reported a study of the relations between certain chemical determinations and the insecticidal effectiveness against houseflies of a number of samples of derris and cube roots. Determinations were made of the optical rotatory power of acetone and benzene extracts of these samples, but the results were not included with the other data. In view of the present interest in this subject, it now seems appropriate to publish this information, together with more recent data on the optical rotatory power of extracts of several additional samples.

FIRST SET OF SAMPLES

METHODS AND RESULTS FOR ACETONE AND BENZENE EXTRACTS

The determination of the toxicity to houseflies of the first set of samples and the computation of a value for "rotenone" based on toxicity have already been described (*9*). This value represents the effectiveness of the root in terms of the toxicity of rotenone to houseflies.

Determinations were made of the optical rotatory power of acetone and benzene extracts of derris and cube roots that had been so prepared that 100 cc was equivalent to 10 g of the original plant material. A saccharimeter and either 1- or 2-dm tubes were used

[1] Received for publication July 3, 1936; issued January 1937.

[2] Reference is made by number (italic) to Literature Cited, p. 838.

for this purpose. Acetone extracts were usually slightly cloudy and often required the use of 1-dm tubes. Benzene extracts of cube root were found to be more highly colored than those of derris, and hence necessitated the use of 1-dm tubes in several cases. The concentration of rotenone equivalent to the rotation was calculated, as described in a previous article (*10*), from the following formula:

$$\text{Concentration (grams per 100 cc of extract)} = \frac{\text{Ventzke reading} \times 0.3466 \times 100}{\text{Length of tube (dm)} \times [\alpha]_D^{20}}$$

For rotenone in acetone $[\alpha]_D^{20} = -105°$. In benzene solution the optical activity of rotenone changes markedly with change in concentration, being represented by the expression $[\alpha]_D^{20} = (1.8 \times \text{concentration} - 233)°$. In the case of the benzene extracts this expression was substituted for $[\alpha]_D^{20}$ in the formula. Since the extracts contained the equivalent of 10 g of plant material per 100 cc, the values obtained were multiplied by 10 to obtain the percentage of "rotenone" in the roots equivalent to the optical rotatory power. The values for optical rotatory power and for equivalent "rotenone", together with the "rotenone" based on toxicity, rotenone by extraction, and total acetone and benzene extracts, are given in table 1.

TABLE 1.—*Comparison between optical rotatory power and other determinations of derris and cube roots. First set of samples*

Sample no.	Material	"Rotenone" based on toxicity	Rotenone by crystallization from CCl_4	Rotenone plus deguelin by Gross-Smith test	Acetone extraction			Benzene extraction			"Rotenone" from optical rotatory power of acetone and benzene extracts	"Toxic value" from rotenone and total benzene extractives
					Total extractives	Optical rotatory power [1] [2]	"Rotenone" from optical rotatory power	Total extractives	Optical rotatory power [1]	"Rotenone" from optical rotatory power		
		Per cent	*Per cent*	*Per cent*	*Per cent*	*Degrees Ventzke*	*Per cent*	*Per cent*	*Degrees Ventzke*	*Per cent*	*Per cent*	*Per cent*
401	Derris root	5.5	0.0	0.8	12.2	+1.4	[3]	9.4	−3.7	2.8	7.0	4.7
523	do.[4]	6	1.1	3.0	12.0	−1.8	3.0	10.4	−6.2	4.6	6.0	5.8
524	do.[5]	11.5	2.0	3.2	22.4	−1.8	3.0	20.0	−10.6	7.9	12.1	11.0
594–B	do	11.5	5.0	14.4	21.2	−5.6	9.2	17.7	−12.7	9.5	9.8	11.4
2217–M–1	do	15	7.8	12.0	24.8	−6.4	10.6	21.2	−15.9	11.9	13.1	14.5
2217–P–1	do	9.5	3.6	5.4	22.4	−1.6	2.6	18.5	−10.9	8.2	12.8	11.0
584	Cube root	10.5	4.1	11.0	18.2	−7.2	11.9	17.3	[2] −14.0	10.5	9.3	9.4
674	do	6	.8	3.9	14.1	−4.6	7.6	13.0	[2] −8.4	6.3	5.2	5.7
686–A	do	18	12.1	18.6	25.4	−11.8	19.5	24.8	−22.4	16.8	14.7	17.2
2218–H–1	do	11	5.6	10.8	21.7	−8.0	13.2	20.3	−16.0	12.0	11.0	11.5
2218–M–1	do	8.5	3.8	9.6	23.0	−5.6	9.2	20.9	[2] −12.2	9.1	9.0	10.6

[1] 100 cc = 10 g of plant material; calculated for 2-dm tube.
[2] Determinations made in 1-dm tube.
[3] Not calculable.
[4] *Derris elliptica.*
[5] *Derris malaccensis.* In other cases species was not identified.

In figures 1 and 2 the values for "rotenone" equivalent to the optical rotatory powers of acetone and benzene extracts, respectively, have been plotted against the values for "rotenone" based on toxicity. In about half the samples the acetone-extract values differed widely from the toxicity values. The benzene extracts gave values much closer to the insecticidal values, but they were in general lower.

COMBINED VALUE BASED ON RESULTS FOR BOTH ACETONE AND BENZENE EXTRACTS

Since the optical activity of rotenone in acetone is quite different from that in benzene, it was thought of interest to calculate the theoretical "rotenone" content from the optical activity of both acetone and benzene extracts and compare such values with the toxicity to houseflies. The optical rotatory power of both acetone and benzene extracts might be considered to be made up of that of rotenone and rotenonelike compounds plus that of dissimilar optically active material. For the purposes of this calculation it might be supposed that the optical rotatory power of all the rotenonelike compounds present is greater in benzene than in acetone to the same degree as is that of rotenone, and that the rotation of the other optically active

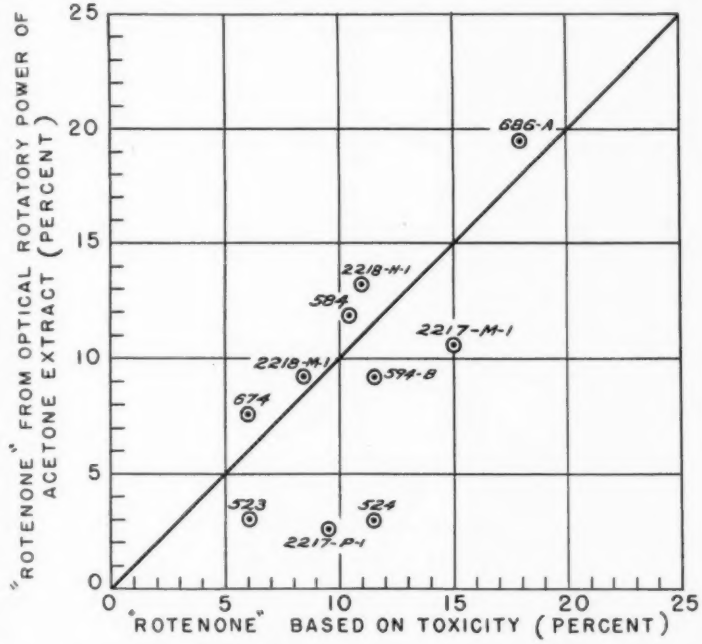

FIGURE 1.—"Rotenone" from optical rotatory power of acetone extracts versus "rotenone" based on toxicity to houseflies.

material is the same in both solvents. For this purpose it must be assumed that acetone and benzene extract the same optically active material from a given sample of root.

The following formula would give such a value for theoretical "rotenone" content:

$$\text{Concentration (grams per 100 cc extract)} = \frac{35.51 - \sqrt{1{,}261 - 9.615}}{(OR_a - OR_b)}$$

where OR_a and OR_b are the observed rotations of acetone and benzene extracts, respectively, in Ventzke degrees in 2-dm tubes. The values

obtained, converted to percentages, are given in table 1. Approximate values could have been obtained by assuming the optical rotatory power of the rotenonelike compounds in benzene to be exactly double that in acetone.

In figure 3 the values calculated from this formula have been plotted against the values for "rotenone" based on toxicity to houseflies. The two sets of values are in better agreement than when calculated separately from the rotatory powers of acetone and benzene extracts. Since little is known of the optical rotatory power of the components of the extract other than rotenone, the calculation is open to question, and the only justification for discussing it is the fair agreement with toxicity values. The agreement with insecticidal value is not so good,

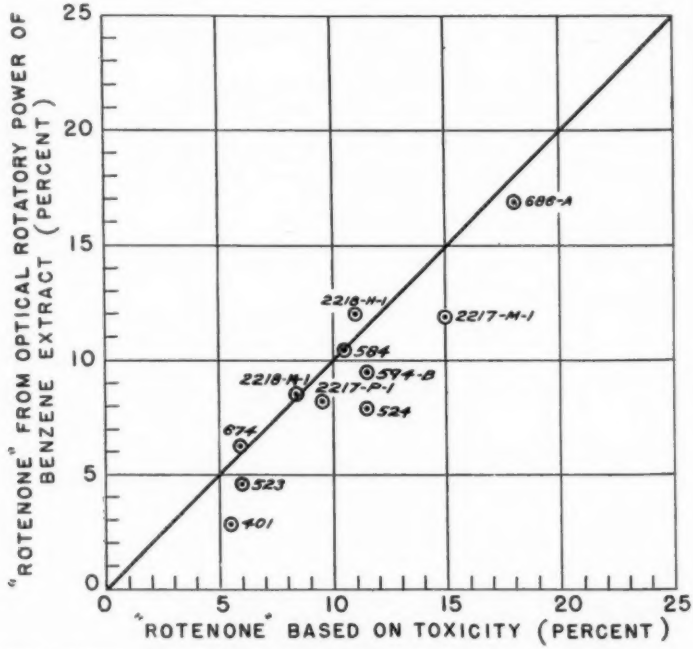

FIGURE 2.—"Rotenone" from optical rotatory power of benzene extracts versus "rotenone" based on toxicity to houseflies.

however, as in the best method referred to in the work already cited (*9, fig. 38, F*).

It might be supposed that the method would be of value as an approximate determination of toxicity, but the calculation recently proposed by Jones and Smith (*11*), based on rotenone and total extractive contents, gives an approximate value more simply and logically than the use of optical activity, which is so largely dependent on unknown constituents. Results for toxic value by this method of calculation, based on rotenone and total benzene extractives, are given in the last column of table 1 for comparison with the values obtained from optical rotation.

DISCUSSION

In table 1 it will be noted that there is no agreement between the rotenone by crystallization and the value obtained from the optical rotatory power of either acetone or benzene extract. Likewise there appears to be no constant relation between the quantity of total extractives and the optical rotation values. In two derris samples and all except one of the cube samples the values obtained from optical rotatory power by all three methods of calculation were approximately in agreement. Since the acetone extract of the derris root containing

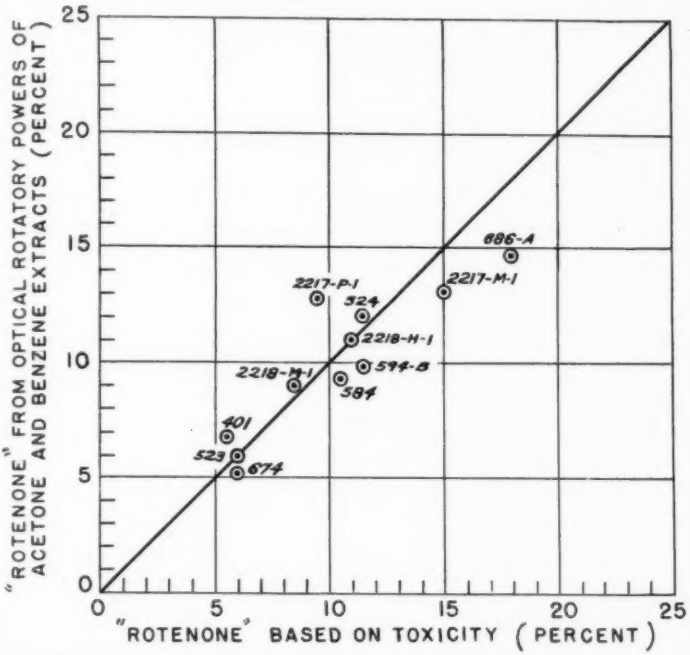

FIGURE 3.—"Rotenone" from combined optical rotatory powers of acetone and benzene extracts versus "rotenone" based on toxicity to houseflies.

no rotenone (sample no. 401) was dextrorotatory, no values for "rotenone" could be calculated.

Danckwortt and his coworkers (*3*) have stated that, since optically active deguelin possesses (according to Takei) about 10 percent as much optical activity in benzene as does rotenone, and since in certain tests to which they refer it is about one-tenth as toxic as rotenone (according to Takei and Gersdorff), the optical rotatory power of the benzene extract should give a good indication of toxicity. This assumes that rotenone and deguelin account for practically all the optical rotatory power, and it is stated by Danckwortt that when 10 percent of the deguelin content is added to the rotenone content the results should agree with the values calculated from benzene rotation. It has already been shown by Haller and LaForge (*7*) that Takei's material was not active deguelin but apparently only a mixture of

inactive deguelin and rotenone. Hence it is not surprising that when the calculation suggested by Danckwortt is applied to the rotenone and the rotenone-plus-deguelin values by the Gross and Smith test in the first series of roots (table 1), the results are widely different from the values obtained from the optical activity of the benzene extracts, as well as the other two optical rotation methods. Furthermore, the toxicity tests to which Danckwortt refers were made on fish rather than insects, and Gersdorff's work was done with optically inactive deguelin which, according to the more recent work of Gersdorff (*6*), and that of Fink and Haller (*4*), might be expected to be less toxic than optically active deguelin.

SECOND SET OF SAMPLES

METHODS AND RESULTS

From the chemical standpoint, for comparison with the results obtained on the first set of samples, it was thought advisable to determine the optical rotatory power of extracts of a number of additional samples of derris and cube roots. For this purpose five samples of derris root containing rotenone, five samples of cube root, and five samples of derris root containing no rotenone were selected. It should be mentioned here that the last five roots, and also no. 401 and no. 524 of the first set, were tested for "hidden" rotenone by the method of Cahn and Boam (*1*). The results of this test and of later determinations by the author's improved crystallization procedure indicate that some of the "no rotenone" samples may contain very small quantities of rotenone, in no case exceeding 0.5 percent. For the purpose of this work, however, the rotenone content of these samples may be considered to be negligible. The optical rotatory powers of acetone and benzene extracts were determined by the method outlined for the first set of roots, and similar calculations were made.

TABLE 2.—*Comparison between optical rotatory power and other determinations of derris and cube roots. Second set of samples*

Sample no.	Material	Rotenone by crystallization from CCl_4	Acetone extraction			Benzene extraction			"Rotenone" from optical rotatory power of acetone and benzene extracts	"Toxic value" from rotenone and total benzene extractives
			Total extractives	Optical rotatory power [1]	"Rotenone" from optical rotatory power	Total extractives	Optical rotatory power [1]	"Rotenone" from optical rotatory power		
		Percent	*Percent*	*Degrees Ventzke*	*Percent*	*Percent*	*Degrees Ventzke*	*Percent*	*Percent*	*Percent*
2710	Derris root	3.3	18.7	[2] −1.8	3.0	15.6	−9.7	7.3	10.9	9.4
2227–R–344	do	6.5	22.0	[2] −5.0	8.2	19.0	−14.0	10.5	12.4	12.8
2793	do	2.9	20.1	[2] −1.4	2.3	17.6	−10.2	7.6	12.1	10.2
2768	do	4.9	19.6	−3.9	6.4	17.8	−12.0	9.0	11.2	11.4
2752	do	5.6	18.9	[2] −2.6	4.3	15.5	−11.7	8.8	12.5	10.6
2708	Cube root	5.2	18.3	−6.6	10.9	17.8	[2] −14.2	10.6	10.4	10.2
2709	do	3.7	16.5	−5.5	9.1	16.4	[2] −12.8	9.6	10.0	8.8
2711	do	1.2	10.7	−2.8	4.6	9.8	[2] −6.0	4.5	4.4	4.6
2779	do	1.7	13.1	[2] −4.6	7.6	12.7	[2] −8.6	6.4	5.5	6.1
2794	do	5.3	18.2	−6.6	10.9	17.2	−14.2	10.6	10.4	10.1
998	Derris root	0	15.2	[2] +2.0	[3]	13.4	−4.7	3.5	9.2	6.7
999	do	0	13.2	[2] +1.6	[3]	11.5	−4.2	3.1	7.9	5.8
1000	do	0	14.4	[2] +1.8	[3]	13.0	−4.8	3.6	9.0	6.5
2288	do	0	18.6	+3.5	[3]	16.2	−5.2	3.9	12.0	8.1
2843	do	0	16.2	[2] +2.4	[3]	13.4	−4.3	3.2	9.2	6.7

[1] 100 cc ⌭ 10 g of plant material, calculated for 2-dm tube.
[2] Determinations were made in 1-dm tube.
[3] Not calculable.

The values for optical rotatory power and equivalent "rotenone," together with the rotenone content by extraction, and total acetone and benzene extractives, are given in table 2. The figures for "toxic value" from rotenone and total extractives are also included to give some comparison between the optical rotatory power and the insecticidal activity in lieu of actual toxicity tests.

DISCUSSION

It will be noted that in the derris roots containing rotenone the sets of values calculated by the three optical rotation methods are quite different. The acetone values are comparable with the actual rotenone content, but the other figures are markedly higher. On the other hand, the values for the cube roots obtained by the three methods are in fair agreement. This tendency was also noted in the first set of cube samples. The agreement might be explained by assuming that all the optically active components are similar to rotenone in exhibiting approximately double the rotatory power in benzene that they do in acetone. In such a case the optically active components of practically all the cube samples would be similar to rotenone in this respect, and hence perhaps very closely related to rotenone. Further, it would follow that most of the derris samples contain materials that are quite unlike rotenone in their optical rotatory relations.

It will be noted that the agreement between the "rotenone" calculated from the combined optical rotatory powers of acetone and benzene extracts and the "toxic value" from rotenone and total extractives is good, indicating that the former values would agree with the actual toxicity to houseflies. For the derris roots containing no rotenone the figures for toxic value are somewhat lower than the combined optical rotation values, as was also true of the derris sample containing no rotenone in the first set of roots.

Acetone extracts of the derris samples containing no rotenone were dextrorotatory although the benzene extracts were levorotatory, as were the derris containing no rotenone (no. 401) in the first set of roots. In these samples the combined values from benzene and acetone extracts were markedly higher than those from benzene alone. It is obvious that these samples contain some dextrorotatory substances. It may be supposed that they also contain some levorotatory material which, like rotenone, has a higher activity in benzene, and thus in the benzene extracts masks the dextrorotatory material. Dextrorotatory components may also be present in other samples of root, particularly those showing poor agreement between the three sets of optical rotation values, but they would be masked in both acetone and benzene by the strong levorotation of the rotenone present. Rowaan (*12*) actually obtained some values by benzene rotation which were lower than the rotenone by crystallization. In no case have the writer's benzene values been below the rotenone content, although the acetone values for a few of the derris samples were lower.

Rowaan has suggested that toxicarol, which he believes to be dextrorotatory, may account in part for these lower values. This might also contribute to the dextrorotation of the writer's "no rotenone" samples in acetone, as well as account for some of the disagreement between the three sets of values for some of the samples. It might

therefore be conjectured that the samples of cube root studied contained little or no toxicarol while most of the samples of derris contained appreciable amounts.

A comparison of the rotenone values obtained by extraction with those obtained by optical rotation, particularly of benzene extracts, shows that in many of the samples there must be materials in addition to rotenone which possess a higher levorotation in benzene at least.

SUMMARY AND CONCLUSIONS

Values for "rotenone" equivalent to the combined optical rotatory powers of both acetone and benzene extracts of derris and cube roots gave an approximate measure of the insecticidal effectiveness of these materials to houseflies. Values calculated from the rotation of benzene extracts did not agree with toxicity so well as did the combined values, and in about half the samples results derived from the optical activity of acetone extracts were widely different from the toxicity values.

Since a method has already been proposed for calculating the approximate toxic value to houseflies of derris and cube roots based on the rotenone and total extractive contents, which is both simpler and less open to question, the use of optical rotatory power cannot be recommended as a means of evaluation.

From the chemical standpoint the results indicate that optically active constituents other than rotenone and deguelin were probably present in the samples of derris and cube tested. Dextrorotatory materials were undoubtedly present in the samples of derris root containing no rotenone, and possibly in other samples. The use of optical rotation should prove of considerable value in further chemical study of the components of extracts of derris and cube roots.

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RELATIVE TOXIC ACTION OF PHENOL AND PHENYL MERCAPTAN, WHEN THE GOLDFISH IS USED AS THE TEST ANIMAL[1]

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INTRODUCTION

In the course of toxicological studies an investigation was made of the toxicity of some of the simpler aromatic substances, beginning with phenol. Since it is known that the replacement of the oxygen atom in the molecule by sulphur frequently increases toxicity, phenyl mercaptan (thiophenol) was selected for a comparative study. The relationship of the two compounds is shown by their structural formulas, $C_6H_5.OH$ and $C_6H_5.SH$.

The boiling point of the sample of phenol used was 183° C., and that of the phenyl mercaptan was 170°. The samples were, therefore, of a high state of purity.

Since rotenone has been used as a standard of comparison in previous studies of a similar nature (*6*, *7*),[2] it was again used as such, and tests were made to establish the resistance of goldfish to this substance.

EXPERIMENTAL PROCEDURE

The method of making the toxicity tests is essentially the same as that previously described (*3*). Goldfish of a single lot, weighing from 3 to 5 g, were used, and a constant temperature of 27° C. was maintained.

Phenol is readily soluble in water to such an extent that aqueous stock solutions could be used. Phenyl mercaptan, however, because of its insolubility, was first dissolved in acetone, and aliquot portions were used to give better dispersion in the test solutions.

Unfortunately, phenyl mercaptan is not completely soluble at most of the concentrations used, as evidenced by the appearance of some minute oily droplets. In the very lowest concentrations these seemed to disappear after a short while, but in others they were replaced by crystals, which proved to be phenyl disulphide. When exposed to oxidizing agents phenyl mercaptan has a tendency to throw off hydrogen and form this compound. This reaction progressed to some extent under the conditions of the tests, but it was found to be relatively slow in the low concentrations if the aliquots were dispersed in the test solutions without undue aeration. In a separate test at a concentration of 10 mg per liter the crystals began to form in about half an hour, but the change was not complete in 4 hours. This solution, as well as those of lesser concentration, became faintly opalescent in 3 to 4 hours. At a concentration of 2 mg per liter there was no apparent opalescence. The toxic action, however, proceeded in an

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[2] Reference is made by number (italic) to Literature Cited, p. 847.

orderly fashion, both as to frequency distributions of survival times at each concentration and as to variation of mean survival time with variation in concentration, even in the range of markedly opalescent solutions. It would appear, therefore, that the toxic effect of the substance is the same whether collodial or in true solution and that the toxic action is not appreciably affected by the partial oxidation of phenyl mercaptan to phenyl disulphide.

Although the concentrations of phenol used were relatively high, the changes in hydrogen-ion concentration caused by its use were well within the range of the variation tolerated by most fishes. Waters having an acidity as high as pH 4.0 may support a good, mixed fish fauna (*2*). A solution of phenol of the highest concentration used has a pH value of 5.7. Wiebe (*9*) has reported that goldfish tolerated a sudden change from water of pH 7.5 to water of pH 6.2. The higher value approximates the average for the city water to which the fish used in this study were acclimated and which was used in making the solutions, and the lower value is that of a solution of 0.28 g of phenol per liter. It would appear, then, that the variation in hydrogen-ion concentration did not introduce a disturbing factor.

DISCUSSION OF TOXIC ACTION

The toxic action of phenol on goldfish is quite different from that of rotenone (*1*, *3*, *4*). The effect of rotenone is one of suffocation through the medium of the destruction of the gill epithelium, and it is a gradual one, there being apparent only a slowly increasing sluggishness. Also there is a comparatively large time tolerance; that is, the survival time required for lethal action at high concentrations is long. In the case of phenol, on the other hand, the fish are strongly affected at once, as shown by a frantic dashing about, and later movements are characterized by spasmodic twitchings. Respiratory movements are slightly increased and become irregular and forced. The fish show no evidence of oxygen hunger as in the case of rotenone poisoning. There is practically no time tolerance. The higher concentrations exert an obviously strong paralytic action, as evidenced by rigid, outstanding fins and locked jaws. This is the type of action produced by nicotine and anabasine (*5*).

In rotenone poisoning the last stage of the physiological action just preceding death, after irritability is lost, is a relatively long one, but in phenol poisoning it is short. In the test to confirm death (dipping the fish into 1:3 hydrochloric acid), with phenol some of the individuals were still capable of muscular movement. Thus, fish killed with phenol retain this ability nearer to the time of death than those killed with rotenone.

Phenyl mercaptan exerts a rapid toxic action like that of phenol, but its insolubility at the higher concentrations required in the tests under the present conditions precludes a definite statement regarding a time tolerance. Despite this, however, there is indicated a time tolerance much smaller than that of rotenone.

TREATMENT OF DATA

Other differences in the action of phenol were soon noted, and because of these peculiarities a study of the frequency distributions was made. As a result of the findings the mathematical treatment

of the data differs from that ordinarily employed in respect to the form of average selected as defining the position of each frequency distribution for the observed survival times. In the tests with rotenone the distributions were nearly normal, the mode corresponding closely with the arithmetic mean. The number of individuals used in a test was insufficient to place the position of the median with stability; however, the average of the middle third closely approximated the average of the whole. Therefore, the arithmetic mean gave the most probable value satisfactorily. On the other hand, in the tests with phenol the frequency distributions were markedly skewed, the degree of skewness increasing rapidly with the decrease in the concentration. Apparently this is due to a strong tendency of individuals to exhibit an increasing resistance to phenol beyond a certain point in the distribution. Thus, a few widely outlying observations in some cases pulled the arithmetic mean far to the right of the mode, and therefore, without the use of a large number of fishes—many more than were permitted by the conditions of the study—the value of this mean was very unstable. The degree of dispersion and the percentage of individuals exhibiting this effect become greater with decrease in concentration, at least to the lowest concentration used. This type of action was also found to be exhibited by nicotine and anabasine, and, since all three compounds exert an obviously strong paralytic action at the stronger concentrations, an asymmetrical distribution of such a high degree of skewness may be common to all compounds of this type of toxic action.

Thus it is seen that the assumption, which is implied by the use of the arithmetic mean, that plus and minus deviations from the mean are equally probable, cannot be justified in this type of toxic action. Frequently at the lower concentrations the longer survival times are more than double the mean. The median is also an unstable value. The mode, although well defined at the higher concentrations, is not so well defined at the lower concentrations where the degree of dispersion becomes great, especially without great increase in the number of observations beyond those permitted in these studies.

The harmonic mean would have the desired property of a lowered relative effect of a few long survival times. It would have, moreover, an easily understandable meaning, for it would simply give, instead of the average survival time at a given concentration, the survival time corresponding to the average velocity of fatality. There is, however, an important objection to its use. Occasionally an individual was found to die much sooner than his companions in a test. This was not due to an obviously poor physical condition, for special precautions were taken to eliminate as far as possible this source of error. Since such an individual appears only occasionally, its effect on the instability of the harmonic mean is considerable. Thus the use of this mean has a disadvantage which it would be desirable to avoid.

In these toxicity tests the magnitudes of the deviations in the various distributions appear to be dependent in some degree on the magnitude of the average. Moreover, the individual observations appear to fall into a geometric rather than an arithmetic series. Therefore, it may be logical to measure variations by their ratio to, rather than their difference from, the average. In this case the geometric mean is the natural average to use.

The study of a number of distributions shows that the frequency curve, if plotted to the logarithms of survival times instead of the observed times, becomes much more symmetrical around the mean, which approaches more closely to the mode (a geometric mode) and therefore the median. For these reasons, in tests with compounds giving such skewed distributions as do the phenols, it appears that the geometric mean defines best the position of the frequency distribution and so will be used in this study. Phenyl mercaptan does not ex-

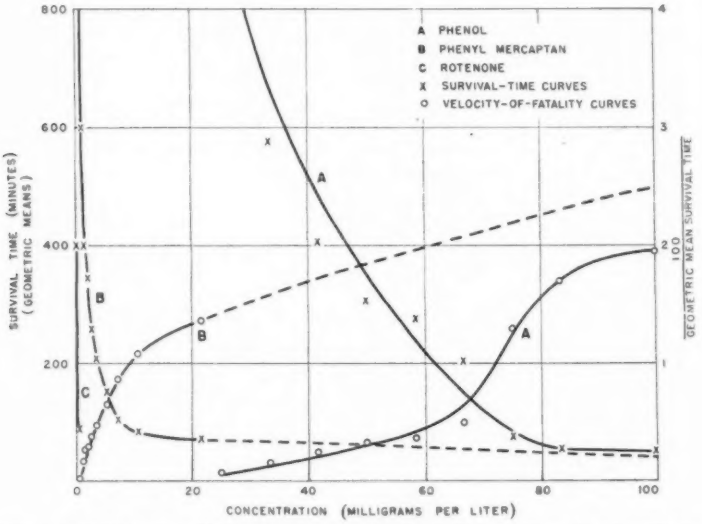

FIGURE 1.—Toxicity curves of phenol, phenyl mercaptan, and rotenone. Rotenone is comparatively so toxic that the features of its survival-curve time cannot be brought out graphically at the scale required to show those of the other compounds.

hibit the degree of skewness that phenol does, its frequency distributions resembling those of rotenone in having but a moderate asymmetry. Since the toxicity of these two compounds is to be compared with that of phenol, however, the results obtained with them are to be treated in the same way.

RESULTS

The toxicological data are given in table 1. The survival-time curves and the velocity-of-fatality curves which are plotted from these data are given in figure 1.

TABLE 1.—*Toxicity of phenol, phenyl mercaptan, and rotenone to goldfish at 27.0° ±0.2° C.*

Concentration (milligrams per liter)	Fishes used	Mean length of fishes	Mean weight of fishes [1]	Mean survival time [2]		100÷geometric mean survival time
				Arithmetic	Geometric	
	Number	*Millimeters*	*Grams*	*Minutes*	*Minutes*	
ROTENONE						
0.500	14	46	2.9	92± 3	90 (×÷ 1.03)	1.11
.300	17	47	3.1	97± 3	96 (×÷ 1.01)	1.04
.200	12	46	2.9	106± 5	103 (×÷ 1.04)	.971
.150	8	53	4.4	138±10	134 (×÷ 1.07)	.746
.100	22	50	3.6	170±11	154 (×÷ 1.06)	.649
.0750	13	55	5.0	215±16	200 (×÷ 1.07)	.500
.0300	10	[3]	[3]	> [4] 400	> [4] 400	< .250
PHENOL						
2,380	4	56	5.3	6	6	17
820	4	53	4.5	9	9	11
311	4	52	4.3	46± 5	43 (×÷ 1.16)	2.33
207	5	55	5.0	39± 3	38 (×÷ 1.10)	2.63
124	6	55	4.9	48± 6	45 (×÷ 1.11)	2.22
99.8	13	47	3.1	53± 3	51 (×÷ 1.06)	1.96
83.2	22	49	3.5	84± 3	59 (×÷ 1.05)	1.69
74.9	8	52	4.3	116± 29	78 (×÷ 1.23)	1.28
66.6	10	54	4.8	231± 26	202 (×÷ 1.10)	.50
58.3	7	56	5.3	315± 43	273 (×÷ 1.16)	.37
50.0	18	54	4.7	478± 83	306 (×÷ 1.16)	.33
41.6	12	56	5.2	624±121	406 (×÷ 1.25)	.25
33.3	12	54	4.8	702± 67	577 (×÷ 1.16)	.17
25.0	10	[3]	[3]	[5] 1,420	> [5] 1,260	< .08
PHENYL MERCAPTAN						
216.0	2	45	2.8	25± 2	25 (×÷ 1.09)	4.00
21.6	2	52	4.3	75± 9	74 (×÷ 1.12)	1.35
10.8	7	57	5.6	86± 4	85 (×÷ 1.04)	1.18
7.19	9	52	4.3	119± 4	117 (×÷ 1.04)	.855
5.39	10	53	4.6	154± 6	152 (×÷ 1.04)	.658
3.59	13	54	4.9	211± 7	208 (×÷ 1.03)	.481
2.70	12	54	4.9	261±11	257 (×÷ 1.04)	.389
2.16	10	56	5.3	349±14	344 (×÷ 1.04)	.291
1.62	11	54	4.9	> [6] 400	> [6] 400	< [6] .250
1.08	9	48	3.4	> [6] 600	> [6] 600	< [6] .167
.54	10	[3]	[3]	> [7] 4,000	> [7] 4,000	< [7] .025

[1] Estimated from length, which measurement excludes the tail.
[2] Errors are probable errors of the means. At the first 2 concentrations of phenol a probable error, if calculated, would have no significance at such short survival times.
[3] Fishes not measured or weighed but of same approximate size.
[4] 1 fish dead in 210 minutes; 1 near death and 8 fishes not near death in 400 minutes, when test was stopped.
[5] 4 fishes died in 850 minutes; 6 still alive after 30 hours, when test was stopped.
[6] Slightly low; temperature not controlled satisfactorily.
[7] Not killed in 66 hours, when test was stopped.

QUANTITATIVE COMPARISON OF RESULTS

A striking difference in the toxic action of phenol and phenyl mercaptan is at once apparent from an inspection of the velocity-of-fatality curves. That of phenol has a sigmoid curvature, signifying that the initial as well as the final acceleration with increase in concentration is relatively slow, whereas that of phenyl mercaptan apparently lacks the initial upward concavity. This is again suggestive of the types of curves obtained with nicotine and rotenone, respectively. Whether this is due to the comparatively large differences in toxicity or to the particular types of action, especially as regards complexity of physiological action, remains to be determined.

To attempt to compare quantitatively such different types throughout their course of action would be meaningless, especially as to thresholds of toxicity or the theoretical velocity of fatality of Powers (*8*). The threshold of phenol cannot be approximated. Under the conditions of the tests, however, the threshold concentration of rotenone is about 0.04 mg per liter, and that of phenyl mercaptan about 0.4 mg per liter. It may be said, therefore, that phenyl mercaptan becomes toxic at much higher concentrations than rotenone (about 10 times as high) whereas phenol becomes significantly toxic (lethal within 3 days) at still higher concentrations than phenyl mercaptan (of the order of 40 times as high). This appears to be a fair comparison from the standpoint of concentration alone.

The insolubility of phenyl mercaptan at the highest concentrations necessary with this particular lot of fish doubtless affects the shape of its curve, and, therefore, any quantitative comparison at this range of the time tolerance would have no general significance.

The compounds may properly be compared, however, at their regions of most efficient action. In these studies this action is a function of concentration and survival time, and a criterion providing such a quantitative comparison has been suggested by the author (*6*). This criterion is the minimum product of concentration and survival time as determined from the survival-time curve, and toxicity under these conditions may be defined as the reciprocal of this product. In the case of rotenone its curve must first be redrawn on an expanded scale. The comparative data are given in table 2. The minimum product of concentration and time is designated by $(ct)_m$; its approximate coordinates, c_m and t_m, are given so that the region of the curve fulfilling this condition can be located readily.

TABLE 2.—*Relative toxicity at 27° C. of phenol, phenyl mercaptan, and rotenone*

Compound	c_m	t_m	$(ct)_m$	Toxicity, $1/(ct)_m$	Relative toxicity according to $(ct)_m$
	Milligrams per liter	*Minutes*	*Gram-minutes per liter*	*Liters per gram per minute*	
Rotenone	0.075	200	0.0150	66.7	1.0
Phenol	85.0	55	4.68	.214	.0032
Phenyl mercaptan	2.00	345	.690	1.45	.022

From these data it is seen that, according to this criterion, phenyl mercaptan is 6.9 times as toxic as phenol but only 0.022 as toxic as rotenone.

SUMMARY

A study has been made of the toxicity of phenol and phenyl mercaptan, and the results have been compared with each other and with those obtained with rotenone. Goldfishes weighing 3 to 5 g each, and of the same lot, were used as the test animals. The observations were made at a constant temperature of 27° C.

The phenolic solutions used were not so acid that the change in hydrogen-ion concentration was a factor to be considered.

Phenol, like nicotine and anabasine, gives markedly skewed frequency distributions, in contradistinction to phenyl mercaptan and rotenone. The geometric mean defines best the position of the distribution in the case of phenol. It differs but little from the arithmetic mean in the case of phenyl mercaptan and rotenone.

Phenyl mercaptan becomes toxic at much higher concentrations than rotenone (about 10 times as high), whereas phenol becomes significantly toxic (lethal within 3 days) at still higher concentrations than phenyl mercaptan (of the order of 40 times as high). Under the conditions of this study the threshold concentration of rotenone is about 0.04 mg per liter and that of phenyl mercaptan is about 0.4 mg per liter. That of phenol, however, cannot be approximated for comparison, since the initial acceleration of its velocity of fatality with increase in concentration is relatively slow.

Phenol has a much more rapid toxic action than rotenone; its effect is immediately apparent, and there is practically no time tolerance at the higher concentrations. Phenyl mercaptan, although not wholly soluble at the higher concentrations required under the foregoing conditions, also has a rapid toxic action.

All the compounds may be significantly compared quantitatively only at their regions of greatest toxic power, of which the minimum product of concentration and survival time is a measure. According to this criterion phenyl mercaptan is seven times as toxic as phenol but only one-fiftieth as toxic as rotenone.

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THE PHYSIOLOGY OF RHIZOPUS ORYZAE [1]

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INTRODUCTION

Several workers have reported the occurrence of lactic acid among the products of the metabolism of glucose by fungi. A survey of the literature and of the work done in this Division on the production of *d*-lactic acid by various species of *Rhizopus* has been made the subject of another paper (*8*).[2] This paper presents a more detailed discussion of the physiology of *Rhizopus oryzae* Went and Geerligs 395 in connection with the production of *d*-lactic acid and fumaric acid.

DESCRIPTION OF ORGANISM

When grown on sterile moistened white bread, this culture makes a dense felt 5 to 7 cm thick of white stolons, at the nodes of which are borne clusters of brown much-branched rhizoids and clusters of 2 to 10 sporangiophores. Sporangiophores are 200μ to 6 mm long by 10μ to 25μ thick. They are brown, erect, branched or unbranched, with smooth walls terminating in a swelling, the apophysis, which with the sporangium forms a sphere. Sporangia are 100μ to 300μ in diameter. They are dark yellowish brown, and their walls are roughened with minute warty protuberances. The columella is spheric to hemispheric, sometimes collapsing, brown, and 70μ to 150μ in diameter. Sporangiospores are angular or irregular in shape, minutely ridged longitudinally, colorless or brown to black in mass, and 5μ to 12μ in diameter. Chlamydospores are elliptic to spheric, or cylindrical, 18μ to 35μ by 10μ to 20μ.

Culture no. 395 in the collection of this Division, of unknown origin, was compared with descriptions of *Rhizopus oryzae* and with numerous cultures received under this name. It is morphologically similar to a culture of *Rhizopus oryzae* supplied by Dr. A. J. Kluyver, University of Delft, Netherlands.

MATERIALS AND METHODS

For storage purposes, cultures were maintained on beer and wort agar slants at 7° C. In order to obtain spores sufficient for the inoculation of a large number of flasks, the organism was transferred to sterile moistened bread. After 4 or 5 days at 30°, this was covered with a felt of stolons, and sporulation was abundant. Some of this mass of stolons and sporangiospores was transferred to a quantity of sterile water. The spores were liberated but vigorous agitation of the suspension, and the stolon mass was then removed by a sterile hook. Counts of the number of spores per cubic centimeter were made with the aid of a hemocytometer, and the suspension was diluted with sterile water to give the desired number of spores per unit volume. One

[1] Received for publication Aug. 17, 1936; issued January 1937.
[2] Reference is made by number (italic) to Literature Cited, p. 857.

cubic centimeter of inoculum was pipetted aseptically into each of the experimental flasks. Since it was found that uniform results could be obtained throughout the range of 12.5 to 100 million spores per flask, some variation in the number of spores per culture was allowed. At the time of inoculation, 5 g of sterile $CaCO_3$ was added to the nutrient solution, unless otherwise stated. For experimental purposes cultures were grown in triplicate in pyrex Erlenmeyer flasks of 200 cc capacity. Ordinarily, the volume of nutrient used was 75 cc per flask. Nutrient solutions were sterilized in an autoclave at 15 pounds steam pressure for 15 minutes. Experimental cultures were maintained at 30° unless otherwise stated.

Throughout most of this work, a commercial grade of glucose monohydrate was employed as the sole carbon source. In one series of cultures, a chemically pure grade of glucose was used. In all cases, the glucose is expressed in terms of anhydrous glucose as determined by the copper reduction method of Shaffer and Hartmann (*7*). Calcium was determined as calcium oxalate by permanganate titration. Fumaric acid was determined by the method described by Hahn and Haarmann (*2*), and lactic acid was determined by the method reported by Friedemann and Graeser (*1*).

RESULTS

COMPARISON OF RHIZOPUS ORYZAE CULTURES GROWN AT 30° AND 40° C.

Cultures were incubated for 15 days, some at 30° and some at 40° C. Data presented in table 1 indicate that cultures maintained at 40° grew more rapidly and contained the larger quantity of calcium in solution at the end of 15 days. However, at the higher temperature the amount of soluble calcium accounted for as the *d*-lactate was less, expressed as percentage of soluble calcium, than in the cultures grown at the lower temperature. Moreover, the total lactic acid produced was less in the culture grown at the higher temperature than in the one grown at the lower temperature. Although no fumaric acid was found in this series of cultures when grown at 30°, a considerable quantity was produced at the higher temperature.

TABLE 1.—*Metabolism of Rhizopus oryzae at 30° and 40° C.*

[75 cc 15-percent glucose; 0.25 g $MgSO_4.7H_2O$, 0.3 g KH_2PO_4, and 2.88 g NH_4NO_3 per liter; 5 g $CaCO_3$ per flask. Duration, 15 days]

Temperature °C.	Weight of mycelium	Glucose consumed	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
			Total	Due to *d*-lactic acid	Due to fumaric acid	Weight	Weight yield [1]	Weight	Weight yield [1]
	Grams	*Grams*	*Grams*	*Percent*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
30	0.352	6.4	0.86	[2] 105	0	4.07	63.5	0	0
40	.947	10.8	1.14	66	17.5	3.38	31.3	.575	5.3

[1] Grams of acid produced divided by grams of glucose consumed.
[2] Approximate.

DURATION OF CULTURE

In order to find when fumaric acid appeared in the culture, triplicate cultures were harvested on the fifth, ninth, thirteenth, seventeenth,

and twenty-first days after inoculation. Data presented in table 2 show rather slow growth and little acid production up to the ninth day, after which the rates of growth and fermentation rose rapidly. The beginning of this period of increased activity immediately preceded the formation of stolons and the onset of sporulation. The observation that fumaric acid appeared at the age of approximately 2 weeks is in general agreement with the work of Kanel (*3*), who reported that although no fumaric acid was present in young cultures it was found in older cultures.

TABLE 2.—*Influence of duration of culture*

[75 cc 15 percent glucose; 0.25 g $MgSO_4 \cdot 7H_2O$, 0.3 g KH_2PO_4, and 2.88 g NH_4NO_3 per liter; 5 g $CaCO_3$ per flask. Temperature, 30° C.]

Age	Weight of mycelium	Glucose consumed	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
			Total	Due to *d*-lactic acid	Due to fumaric acid	Weight	Weight yield [1]	Weight	Weight yield [1]
Days	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
5	0.040	1.3	0.067			0.060			
9	.039	2.0	.24	87	0	.93	46.5	0	0
13	.220	6.3	.85	88	0	3.36	53.3	0	0
17	.889	10.5	1.28	78	8.7	4.50	42.9	.314	3.0
21	1.032	11.2	1.44	72	13.9	4.66	41.6	.564	5.0

[1] Grams of acid produced divided by grams of glucose consumed.

Data presented in table 2 show that *Rhizopus oryzae* did not utilize the *d*-lactic acid during the later stages of the fermentation. Further experiments have given the same result, even though the cultures were incubated for 49 days and no glucose was present in the flasks during the last 20 days of this period.

NUTRITION

Effect of Varying the Glucose Concentration of the Nutrient Medium

The glucose content of the nutrient solution was increased from 10 to 30 percent by additions of approximately 5 percent. Data presented in table 3 indicate that a glucose concentration of 15 percent was most favorable, as measured by the weights of the mycelia, the consumption of glucose, and the formation of lactic acid. It is worthy of note that no fumaric acid was found in the culture solutions having the highest original glucose content.

TABLE 3.—*Effect of varying the glucose concentration*

[75 cc nutrient solution contained 0.25 g $MgSO_4 \cdot 7H_2O$, 0.3 g KH_2PO_4, and 1.44 g NH_4NO_3 per liter; 5 g $CaCO_3$ per flask. Duration, 14 days. Temperature, 30° C.]

Glucose concentration	Weight of mycelium	Glucose consumed	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
			Total	Due to *d*-lactic acid	Due to fumaric acid	Weight	Weight yield [1]	Weight	Weight yield [1]
Percent	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
9.6	0.293	6.8	1.05	84.7	3.8	4.00	58.8	0.12	1.8
15.1	.324	8.5	1.26	87.4	3.5	4.96	58.4	.13	1.5
20.1	.266	7.6	1.06	86.6	3.2	4.14	54.5	.10	1.3
24.9	.044	3.0	.29	83.0	[2]	1.10	36.7	[2]	
30.0	.129	5.4	.49	88.0	0	1.95	36.1	0	0

[1] Grams of acid produced divided by grams of glucose consumed. [2] Trace.

Effect of Varying the KH_2PO_4 Concentration of the Nutrient Medium

Cultures were made which contained 0.15, 0.3, 0.6, and 1.2 g of KH_2PO_4 per liter. Approximately 15 percent of glucose, 0.25 g of $MgSO_4.7H_2O$ per liter, and 1.44 g of NH_4NO_3 per liter were used in each. Data presented in table 4 show that although the differences were not great, the cultures with the two highest quantities of KH_2PO_4 were most active in the consumption of glucose and the formation of *d*-lactic acid. At these two concentrations, the percentage of the soluble calcium accounted for as calcium *d*-lactate was also greatest.

Table 4.—*Effect of varying the KH_2PO_4 content of the nutrient medium*

[75 cc nutrient solution contained approximately 15 percent, glucose; 0.25 g $MgSO_4.7H_2O$ and 1.44 g NH_4NO_3 per liter; 5 g $CaCO_3$ per flask. Duration, 15 days. Temperature, 30° C.]

KH_2PO_4 per liter (grams)	Weight of mycelium	Glucose consumed [2]	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
			Total	Due to *d*-lactic acid	Due to fumaric acid	Weight	Weight yield [1 2]	Weight	Weight yield [1 2]
Grams	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
0.15	0.397	8.1	1.24	78.5	8.1	4.38	54.1	0.28	3.5
.30	.416	8.2	1.21	81.7	7.4	4.44	54.1	.26	3.2
.60	.419	9.2	1.41	86.4	7.1	5.47	59.5	.28	3.0
1.20	.372	8.9	1.36	85.5	4.4	5.25	59.0	.18	2.0

[1] Grams of acid produced divided by grams of glucose consumed. [2] Approximate.

Effect of Varying the NH_4NO_3 Concentration of the Nutrient Medium

Nitrogen was supplied to a series of cultures as NH_4NO_3 in the quantities of 0.75, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, and 12.0 g of NH_4NO_3 per liter. Data presented in table 5 show that the optimum range of concentration of NH_4NO_3 for growth as measured by the weight of the mycelia was 3.0 to 6.0 g per liter. The range most favorable for the formation of *d*-lactic acid was 1.5 to 6 g per liter. However, better yields of lactic acid, based on the glucose consumed, were obtained at the lowest and highest concentrations than at the concentrations which were optimal for the growth of the fungus and the production of *d*-lactic acid in quantity. Table 5 also shows that at the higher concentrations of NH_4NO_3 (7.5 to 12 g per liter) fumaric acid was not found. At all the lower concentrations fumaric acid was found.

Table 5.—*Effect of varying the NH_4NO_3 concentration of the nutrient medium*

[75 cc 13.2-percent glucose nutrient solution contained 0.25 g $MgSO_4.7H_2O$ and 0.3 g KH_2PO_4 per liter; 5 g $CaCO_3$ per flask. Duration, 17 days. Temperature, 30° C.]

NH_4NO_3 per liter (grams)	Weight of mycelium	Glucose consumed	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
			Total	Due to *d*-lactic acid	Due to fumaric acid	Weight	Weight yield [1]	Weight	Weight yield [1]
Grams	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
0.75	0.134	5.6	0.99	88.5	5.3	3.94	70.4	0.150	2.7
1.5	.290	6.9	1.08	88.8	4.4	4.34	62.9	.140	2.0
3.0	.656	8.0	1.17	82.0	4.3	4.49	56.1	.155	1.9
4.5	.744	8.1	1.11	86.5	4.2	4.29	53.0	.135	1.7
6.0	.767	7.6	1.07	87.5	(2)	4.22	55.5	(2)	
7.5	.233	4.8	.78	88.4	0	3.09	64.4	0	0
9.0	.145	3.3	.58	89.5	0	2.35	71.2	0	0
12.0	.214	2.2	.67	80.5	0	2.42	[3] 110.0	0	0

[1] Grams of acid produced divided by grams of glucose consumed. [2] Trace. [3] Approximate.

Use of Various Nitrogen Sources by Rhizopus oryzae

$NaNO_3$, $NaNO_2$, $(NH_4)_2SO_4$, NH_4Cl, *d*, *l*-alanine, *d*-glutamic acid, glycine, and peptone were supplied, each as the sole source of nitrogen, in quantities equivalent to 0.5 g of nitrogen per liter. NH_4NO_3 and (inadvertently) urea were supplied in quantities equivalent to 1 g of nitrogen per liter. It had been found that not 1 of about 50 cultures studied was able to utilize nitrate nitrogen. The ammonium nitrogen present in the NH_4NO_3 supplied was equivalent to the total nitrogen supplied in the other nitrogen sources. When $NaNO_3$ was supplied no growth occurred; when $NaNO_2$ was supplied, scant growth, averaging 0.026 g per flask, occurred. Data presented in table 6 show that all the other sources of nitrogen supplied were fairly satisfactory. It is significant that this organism was able to utilize ammonium and amino nitrogen, but not nitrate nitrogen, and it utilized nitrite nitrogen very poorly.

Table 6.—*Effect of different nitrogen sources on the metabolism of Rhizopus oryzae*

[75 cc 15.1-percent glucose nutrient solution contained 0.25 g $MgSO_4 \cdot 7H_2O$ and 0.3 g KH_2PO_4 per liter; 5g $CaCO_3$ per flask. Duration, 14 days. Temperature, 30° C.]

Nitrogen source		Weight of mycelium	Glucose consumed	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
Formula or name	Quantity per liter			Total	Due to *d*-lactic acid	Due to fumaric acid	Weight	Weight yield [1]	Weight	Weight yield [1]
	Grams	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
$NaNO_3$	3.00	0.000	0.0	0.00	0.0	0.0	0.00	0.0	0.000	0.0
$NaNO_2$	2.56	.026								
NH_4NO_3	2.88	.596	8.1	1.05	86.0	3.2	4.03	49.8	.099	1.2
$(NH_4)_2SO_4$	2.36	.301	6.2	.79	95.0	3.5	3.36	54.2	.082	1.3
NH_4Cl	1.98	.370	7.2	1.05	87.5		4.13	57.4		
Urea	2.14	.613	8.6	1.24	87.5	6.0	5.44	63.3	.217	2.5
d, *l*-alanine	3.30	.350	8.3	1.26	86.0	8.6	4.87	58.7	.312	3.8
d-glutamic acid	5.46	.356	7.1	1.08	83.0	6.6	4.05	57.0	.206	2.9
Glycine	2.67	.257	5.9	.87	86.0	7.2	3.37	57.1	.183	3.1
Peptone	3.24	.219	8.3	1.29	85.0	5.0	4.95	59.6	.189	2.3

[1] Grams of acid produced divided by grams of glucose consumed.

Effect on Metabolism of Rhizopus oryzae of the Addition of Zinc, Iron and Chromium Salts to the Nutrient Medium

Increased metabolic activity of various micro-organisms when salts of zinc, iron, or chromium were added to the nutrient media have been observed by various investigators (*4*, *5*, *6*). Accordingly, cultures were made to which 20 mg per liter of Zn, Fe, and Cr were added in the form of their salts as $ZnSO_4 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, and $Cr(NO_3)_3 \cdot 6H_2O$. The basic nutrient solution contained 14.2 percent of chemically pure glucose, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of KH_2PO_4, and 2.88 g of NH_4NO_3 g per liter. Five grams of $CaCO_3$ per flask was added to half the flasks of each group. There was no increased glucose consumption in the presence of any of the metallic ions when $CaCO_3$ was not supplied, but when it was supplied there was a marked increase in glucose consumption in the $ZnSO_4$ cultures, as well as an increase in the quantity of *d*-lactic acid produced. However, no difference resulting from the addition of $CaCO_3$ to the cultures which contained chromic or ferric ions was apparent. Accordingly, the effect of zinc on the metabolism of *Rhizopus oryzae* was further investigated. To a

series of cultures containing the same basic nutrient salt medium and 15.4 percent of commercial glucose, $ZnSO_4$ was added so that the zinc ion was present in quantities of 0.1, 1, 10, 20, 50, and 100 mg per liter. The data in table 7 show that the addition of $ZnSO_4$ to the culture medium exerted a marked influence on the growth of *R. oryzae* as measured by the weights of the mycelia, the consumption of glucose, and the production of *d*-lactic acid. The concentration of Zn of 10 to 100 mg per liter was most satisfactory for growth and glucose consumption. While the greatest production of *d*-lactic acid expressed in absolute quantity occurred at the Zn concentration of 10 mg per liter, when expressed as weight yields based on the glucose consumed, the percentage yields of *d*-lactic acid were progressively less as the Zn concentration increased.

TABLE 7.—*Effect of the addition of various quantities of $ZnSO_4$ to the nutrient medium*[1]

75 cc 15.4-percent glucose nutrient medium contained 0.25 g $MgSO_4 \cdot 7H_2O$, 0.3 g KH_2PO_4, and 2.88 g NH_4NO_3 per liter; 5 g $CaCO_3$ per flask. Duration, 11 days. Temperature, 30° C.]

Zinc concentration per liter (milligrams)	Weight of mycelium	Glucose consumed	Calcium dissolved		*d*-Lactic acid	
			Total	Due to *d*-lactic acid	Weight	Weight yield[2]
	Grams	*Grams*	*Grams*	*Percent*	*Grams*	*Percent*
0.0	0.131	3.7	0.50	92	2.08	56.2
0.1	.189	3.5	.47	[3] 106	2.24	64.0
1.0	.316	4.6	.63	[3] 105	2.96	64.2
10.0	.830	8.3	.73	[3] 103	3.40	41.0
20.0	.807	7.8	.71	93	2.97	38.1
50.0	.804	8.3	.63	89	2.52	30.4
100.0	.669	7.7	.65	86	2.52	32.7

[1] No fumaric acid was produced.
[2] Grams of acid produced divided by grams of glucose consumed.
[3] Approximate.

FERMENTATION OF GLUCOSE BY FINISHED MATS OF RHIZOPUS ORYZAE UNDER DIFFERENT CONDITIONS OF NITROGEN NUTRITION

Data in table 2 show that fumaric acid is not produced during the first days of growth of the organism, and data in table 5 show that the production of fumaric acid may be suppressed by increasing the NH_4NO_3 concentration of the medium beyond 6 g per liter. With these facts in mind, cultures were made in which were used approximately 15 percent of glucose, and 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of KH_2PO_4, and 2.88 g of NH_4NO_3 per liter. After 17 days the solutions were drained from these cultures, and three series of triplicate cultures were established by the addition of 50 cc each of new culture solutions. One was similar to the original culture medium, another contained no nitrogen source, and in a third NH_4NO_3 was present at a concentration of 9 g per liter. Further harvests and replacements of solutions were made at 5-day intervals. Between the harvest of the culture medium and the replacement with a new nutrient solution the mycelial pads were floated on sterile distilled water for a short time to wash out any adherent glucose or metabolic products. Five grams of sterile $CaCO_3$ was added dry beneath the mats before the replacement solutions were supplied. The control cultures retained the original solutions throughout the 32-day period. Data

presented in table 8 show that high concentrations of nitrogen, even in the matured cultures, were unfavorable for the production of fumaric acid. Likewise, the nitrogen-starvation condition was unfavorable for fumaric-acid production. When the nitrogen supply was 2.86 g NH_4NO_3 per liter, the formation of fumaric acid depended on the maturity of the mycelium.

TABLE 8.—*Fermentation of glucose by finished mats of Rhizopus oryzae under varying conditions of nitrogen nutrition*

Nutrient solution contained 0.25 g $MgSO_4 \cdot 7H_2O$ and 0.3 g KH_2PO_4 per liter; 5.0 g $CaCO_3$ per flask. Temperature 30° C.]

NH_4NO_3 per liter (grams)	Age of mat	Glucose supplied	Glucose consumed	Calcium dissolved			*d*-Lactic acid		Fumaric acid		Weight of mycelium	Volume
				Total	Due to *d*-lactic acid	Due to fumaric acid	Weight	Weight yield [1]	Weight	Weight yield [1]		
	Days	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*	*Grams*	*Cc*
2.86	17	11.4	8.55	1.00	101	0.0	4.57	53.8	0.000	0.0	.....	75
0.00	22	7.5	6.2	.75	79.0	10.8	2.65	42.7	.236	3.8	.....	50
0.00	27	8.65	5.2	.63	76.0	8.4	2.17	41.7	.155	3.0	.....	50
0.00	32	7.5	3.6	.55	76.5	8.1	1.87	51.9	.129	3.6	0.885	50
2.86	22	7.5	6.7	.80	75.0	9.1	2.71	40.4	.211	3.1	.....	50
2.86	27	8.65	7.4	.72	57.0	27.9	1.84	24.9	.582	7.9	.....	50
2.86	32	7.5	7.0	.79	49.5	34.2	1.74	24.9	.780	11.1	2.631	50
9.00	22	7.5	7.3	.69	80.0	(2)	2.48	34.0	(2)	.....	.....	50
9.00	27	8.65	8.3	.55	60.0	9.6	1.47	17.7	.154	1.9	.....	50
9.00	32	7.5	7.5	.47	66.0	.0	1.40	18.7	.000	.0	3.666	50
Control	32	11.4	11.4	1.23	84.5	7.3	4.69	41.1	.260	2.3	1.024	75

[1] Grams of acid produced divided by grams of glucose consumed. [2] Trace.

DISCUSSION OF RESULTS

It will be noted that throughout this work the total acidity, as measured by calcium in solution, was never completely accounted for by lactic and fumaric acids. The preponderance of lactic acid in these solutions adds considerably to the difficulty of analysis, and at present the authors are not prepared to give an accurate and complete accounting for the total acidity. However, positive Denigès' tests for malic acid have been obtained on some of the solutions, and extremely small quantities of acetic acid have been isolated from certain liquors. It is probable that small amounts of succinic acid are also present. In addition to the five acids, *d*-lactic, fumaric, *l*-malic, acetic, and succinic, alcohol has been detected among the products of the glucose metabolism of *Rhizopus oryzae*.

The data presented in tables 1, 2, 3, 5, and 8 show that the production of fumaric acid varies and is governed to some extent by the cultural conditions and composition of the medium. Data in tables 2, 3, and 5 show that fumaric acid formation is suppressed by increased glucose concentration and by increased NH_4NO_3 concentration and is not found in cultures until after 13 days' incubation. Although table 1 shows no fumaric acid produced at 30° C., occasionally cultures of similar age were found throughout the course of this work in which fumaric acid was produced at 30°. However, in fumaric-acid production, cultures grown at 40° always showed marked superiority over those grown at 30°. The fact that fumaric acid was formed under similar conditions of nutrition in one series of cultures and not in another may have been due to differences in age or relative maturity of the mycelia.

In the section on the duration of culture it was reported that shortly after the thirteenth day the organism passes from the purely vegetative growth phase to the reproductive. This follows a sharp drop in the quantity of glucose remaining in the culture solution. It is doubtful whether sporulation is a reaction of starvation, for its occurrence corresponds with the greatest increase in weight of the mycelium. However, it should be borne in mind that the mycelium formed at this time is composed of stolons, which may be considered as accessory reproductive organs and probably play no part in the fermentation. Therefore, the pad of mycelium must be separated into two parts for interpretation of its metabolism. The first is the actively fermenting layer, which is in direct contact with the solution and which gives rise, after the fermentation has made considerable progress, to the second type of mycelium, the stolons. When $ZnSO_4$ is added to the nutrient medium, the formation of the second type of mycelium is hastened, so that sporulation occurs on the third day, when vigorous fermentation is just beginning.

That $ZnSO_4$ (20 mg Zn per liter) seriously alters the metabolism of the fungus is still further indicated by the way in which the yields of *d*-lactic acid decrease as the zinc concentration increases. At the lower concentration of zinc, sporulation is initiated later than at the concentration of 20 to 100 mg per liter.

The $CaCO_3$ used in the experiments reported here contained enough magnesium to support considerable fungus growth. Since, by adding $CaCO_3$, an undetermined quantity of magnesium was added to each culture, no investigation of the influence of magnesium on the metabolism of *Rhizopus oryzae* was made.

SUMMARY

Factors influencing the production of dextro-lactic acid, fumaric acid, and the metabolism of *Rhizopus oryzae* have been investigated.

The temperature of 40° C. was more favorable for growth and glucose consumption than was that of 30°, but less favorable for *d*-lactic acid production.

The period of greatest activity in *d*-lactic acid production immediately preceded sporulation.

The glucose concentration most favorable for the production of *d*-lactic acid was approximately 15 percent.

Slightly greater yields of *d*-lactic acid were obtained when the KH_2PO_4 concentration was 0.6 or 1.2 g per liter than when the concentrations were lower.

Fumaric acid production was suppressed in cultures containing more than 6 g of NH_4NO_3 per liter. A wide range of NH_4NO_3 concentration, 1.5 to 6.0 g per liter, was favorable for *d*-lactic acid production.

Rhizopus oryzae readily utilized NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, urea, *d*-, *l*-alanine, *d*-glutamic acid, glycine, and peptone as nitrogen sources. $NaNO_2$ was an unsatisfactory nitrogen source, and when $NaNO_3$ was supplied as the sole source of nitrogen, no growth occurred.

In the presence of $CaCO_3$, $ZnSO_4$ exerted an influence on the metabolism of *Rhizopus oryzae*, the greatest quantity of *d*-lactic acid being produced at the concentration of 10 mg per liter of zinc.

Under favorable conditions of nitrogen metabolism, the formation of fumaric acid depended on the maturity of the mycelium.

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CHEMICAL COMPOSITION AND FERMENTATION STUDIES OF CITRON[1]

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INTRODUCTION

The citron, *Citrus medica* Linn., originally introduced into southern Europe from the Orient, is now grown in limited quantities in Puerto Rico, California, and Florida. When ripe the fruit resembles a giant lemon. It is normally picked while it is somewhat immature and the epicarp is still green. The essential oils in the fruit are oil of citron and oil of cedra, which are present in oil sacs in the skin (fig. 1). The thick mesocarp is used for preserving; the pulpy center containing the seeds is discarded.

The true citron should not be confused with the citron melon, a variety of *Citrullus vulgaris*, the common watermelon. The true citron is inedible in the raw state. In preparation for preserving it is usually halved and subjected to a prolonged fermentation in either sea water or a 5- to 10-percent salt brine. The fermentation removes the bitterness, clears the mesocarp, softens the tissues, and makes it possible for them to absorb high concentrations of sugar. Following the removal of salt from the peel, the preserving process consists essentially in gradually increasing the sugar content of the peel by immersion in a series of sugar sirups of increasing strength. After draining and drying, the peel is ready for market under the name of either preserved or candied citron.

Because of the paucity of published data on the fermentation and the chemical and nutritive properties of citron, a laboratory study involving these subjects seemed desirable. A review of the literature showed no analyses of fresh citron, few determinations of the mineral constituents of preserved citron, and no data at all on the vitamins. Furthermore, the findings of Chadefaux (*3*)[2] and Hollande and Chadefaux (*8*) on the fermentation of Corsican citron appeared not to have been substantiated by other investigators.

EXPERIMENTAL MATERIAL AND METHODS

Fresh unripe citrons were received biweekly from Puerto Rico. The fruit when stored kept perfectly for several weeks at a temperature of 35° F. Chemical analyses and vitamin A and C determinations were made on both the fresh and the preserved citrons. In order that comparable results might be obtained, analyses were made on the same lot of citrons before and after preserving.

FERMENTATION OF CITRON

The first step in the commercial curing process is to place the longitudinally cut halves or caps of unripe citron into tuns or hogs-

[1] Received for publication Dec. 9, 1935; issued January 1937. Contribution no. 231 of the Massachusetts Agricultural Experiment Station.

[2] Reference is made by number (*italic*) to Literature Cited, p. 866.

heads filled with either sea water or a 5- to 8-percent common salt solution. At outdoor temperatures the strength of the brine is renewed after about 15 days because of dilution. After 40 to 50 days, when the caps are transparent and cured, the brine is made up to approximately 10 to 12 percent of salt for shipping and long storage. Citron thus treated is imported into the United States from Italy, Greece, and Corsica.

The experimental fermentation took place at room temperature in large crocks containing a 5-percent solution of sodium chloride

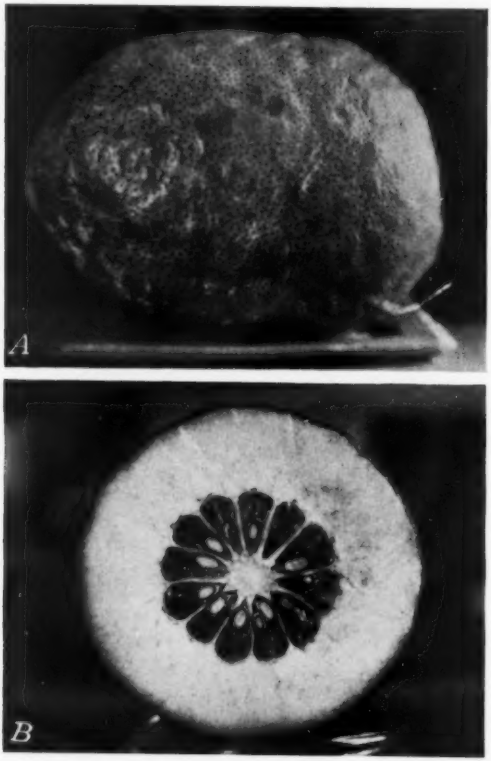

FIGURE 1.—*A*, A fresh green citron showing the prominent oil sacs in the epicarp; *B*, cross section of a citron showing the fleshy mesocarp used for preserving.

covered with a layer of paraffin. The strength of the brine was renewed after 15 days, and after 35 days the citron caps were translucent, clear green, and showed no opaque spots. The fermentation was entirely normal. The characteristic yeasts and bacteria isolated by Hollande and Chadefaux (*8*) were found in large numbers in the brine. Scum yeasts were also plentiful after 10 to 15 days, but were kept under control by the paraffin layer at the surface. The acidity of the brine increased steadily from 0 at the beginning of the experiment to 7 g of acid calculated as acetic per liter after 14 days. Lactic acid was

present as shown by qualitative tests. After 14 days the acidity remained nearly constant, although a decline to 6 g per liter was observed in some crocks. This was probably due to the destruction of acid by scum yeasts. In general, the rise in acidity coincided well with increased counts of yeasts and bacteria. The latter increased steadily for 20 days. The yeasts seemed to reach a maximum at about 10 to 15 days; after this period counting was difficult because of the presence of scum yeasts and debris. Both direct counts of total cells and plate counts of viable cells were made. The direct counts far outnumbered the agar plate counts. Detailed cultural studies of the micro-organisms in the fermenting brine fully confirmed the findings of Hollande and Chadefaux (*8*) and of Chadefaux (*3*) as to the character of the micro-organisms. These were designated *Saccharomyces citri medicae* and *Bacillus citri medicae*. Thus it appears that the fermentation of Puerto Rico citron is very similar to that of Corsican citron, and is due to the same organisms. The fermented citron soon softens excessively and loses its characteristic flavor and color unless the concentration of the brine is raised to 10 or 12 percent. At cool temperatures well-brined citron will keep for 2 years or more. The use of sulphur dioxide (SO_2) or sodium sulphite (Na_2SO_3) in sufficient concentrations to give 200 to 500 parts per million of SO_2 is also useful for preserving the fermented citron in brine. Even citron in 5-percent brine may be thus perfectly preserved. The SO_2 is readily removed along with the salt in the freshening process.

PRESERVING THE FERMENTED CITRON

The final manufacturing process consists of first desalting the citron halves by boiling them in twice their weight of water until the flesh is tender. The citrons then are soaked in running water for several hours. The salt content is reduced to 1 to 2 percent. The peel is translucent, semisoft, and free from opaque spots. The pulpy centers are removed by hand and discarded, and the citrons are then preserved by gradually building up, over a period of several days, the sugar concentration of the flesh by the use of hot sirups. The first sirup contains 15 to 20 percent of sugar, the final one about 75 percent. The sugars used are usually mixtures of cane or invert sugars and glucose. After the sirup has been drained off the citron is dried in a hot-air kiln until the moisture content is reduced to 18 to 25 percent. It is then ready for packing or dicing for the retail trade. There are many variations of the foregoing method but the resulting products are very similar. Candied or glacé citron is prepared by simply dipping the peel in boiling supersaturated sucrose sirup. General data on brining and candying of citron are given by McCulloch (*11*), Cruess and Glickson (*5*), and Chace (*2*). The preserving sirups used in this study contained 65 percent of sucrose and 35 percent of glucose sirup, and the preserved citron contained 70 to 74 percent total sugars (sucrose and glucose). The final moisture content of the preserved peel was between 17.0 and 19.5 percent. The preserving process took 10 days. The peel was dried for 12 hours at 120° F. in a current of air. Sample lots of peel were also preserved by using pure sucrose and different percentages of sucrose and glucose sirup. Some of the lots were also dried to various moisture contents which ranged from

17 to 25 percent. The pure sucrose gave a peel that was too firm in texture and of poor keeping quality because of sugar crystallization. The use of glucose sirup in the preserving process greatly improved the quality of the finished peel. There was no mold or fermentation on peel having a moisture content of 17.0 to 18.5 percent, even after long storage.

CHEMICAL COMPOSITION OF FRESH GREEN AND PRESERVED CITRON

The methods of the Association of Official Agricultural Chemists (*1*) were followed in making the analyses. Copper was determined by the method of Lindow, Elvehjem, and Peterson (*10*), iron according to Stugart (*18*), and iodine by Morse's modification of the Von Fellenberg method (*12*). The results of the analyses are shown in table 1, and because of the scarcity of published data, several partial analyses found in the literature are also included. The pulpy portion of the fruit, including the seeds, was removed from all samples before analysis; the results therefore represent the edible portion only.

The moisture content of citron is somewhat higher than that of other citrus fruits, and the ash, protein, and ether extract are about the same. In spite of the firm mesocarp, the citron is very low in crude fiber and must consist largely of digestible carbohydrates rather than lignin. There is not as much difference between the unripe fresh fruit and the preserved fruit as might be supposed. The total sugars as invert in the fresh fruit are rather low, only 1.55 percent, though the total carbohydrates (by difference) are fairly high, that is, 9.41 percent.

TABLE 1.—*Chemical composition of fresh green and preserved citron*

Determination	Data from this investigation			Data from Sherman (*15*), preserved fruit	Data from König (*9 Bd. 1, p. 912*), preserved fruit	Data from Hartel and Kirchner(7), preserved fruit
	Fresh unripe fruit	Preserved fruit	'Commercially preserved product			
Moisture ..... percent	88.56	18.81	17.00	19.0	2.91	.....
Ash ..... do	.44	.48	.51	.9	.....	.....
Protein (N×6.25) ..... do	.15	.16	.18	1.5	.....	.....
Ether extract ..... do	.32	.33	.38	1.5	.....	.....
Crude fiber ..... do	1.09	1.37	1.89	.....	3.69	.....
Total sugars (invert) ..... do	1.55	43.05	45.81	.....	30.90	.....
Glucose ..... do	.....	28.14	32.36	.....	29.87	11.5–30.8
Sucrose ..... do	.....	14.91	13.45	.....	1.01	14.3–50.0
Total carbohydrates (except fiber) ..... percent	9.41	78.85	72.60	78.1	.....	.....
Calcium oxide ..... do	.09	.06	.12	.17	.....	.....
Phosphorus pentoxide ..... do	.046	.038	.05	.151	.....	.....
Potassium oxide ..... do	.114	.021	.....	.51	.....	.....
Ferrous oxide ..... p. p. m.	9	4.1	16	.....	.....	.....
Cupric oxide ..... do	1	1.7	6	.....	.....	.....
Iodine ..... p. p. b.	3	(1)	(1)	.....	.....	.....
Alkalinity of ash [2]	6.9	8.3	7.0	9.8	.....	.....
Vitamin A [3] ..... cc units	0.8–1.0	(4)	.....	.....	.....	.....
Vitamin C [5] ..... do	6	(1)	.....	.....	.....	.....

[1] Trace.
[2] Cubic centimeter of normal HCl required to neutralize the ash from 100 g.
[3] International units per gram; 1 Sherman unit of vitamin A = 1.4 international units.
[4] Less than 1.
[5] 1 international unit of vitamin C = 0.05 mg l-ascorbic acid. Protective level for guinea pigs was determined to be 0.5 mg l-ascorbic acid.

A partial mineral analysis is given for both the fresh and preserved citron. As in other citrus fruits, the ash is definitely alkaline and there are appreciable amounts of calcium, phosphorus, and copper. The iron and iodine content is low. The increased amount of iron and copper in some of the preserved samples is probably due to contamination by metals during the manufacturing process. The presence of copper salts in minute quantities causes a noticeable greening of the epicarp. The presence of vitamins A and C is noted in table 1 for the sake of completeness. The vitamin studies are reported below.

VITAMIN C IN FRESH GREEN CITRON AND IN FERMENTED PEEL

The assay for vitamin C was conducted by the method of Sherman, LaMer, and Campbell (*17*), 300-g guinea pigs being fed for 90 days. The vitamin C-free ration kept before the animals at all times consisted of baked skim milk 30 parts, butter fat 10 parts, bran 29.5 parts, rolled oats 29.5 parts, sodium chloride and cod-liver oil, each 1 part. The animals were housed in individual wire cages and were fed at five levels of green citron and three levels of cured, fermented citron. Since guinea pigs dislike sweet foods, they were first fed the desalted fermented citron to determine whether any vitamin C remained in the cured fruit. Had some been found, it was intended to next assay the preserved citron. However, inasmuch as only a trace of vitamin C was found in the cured fermented product before the addition of sugar, there was no use in assaying the finished product, i. e., the preserved or candied citron. The desalting was accomplished by boiling the brined peel in water for 1 hour. The ratio of peel to water by weight was approximately 2 to 1.

Sectors of green citron, exclusive of pulp and seeds, were fed in these tests. The animals ate both the green and the cured citron without forced feeding. Three guinea pigs were fed at each level. Average weight gains and scurvy scores at autopsy are shown in figure 2.

The graphs in figure 2 show clearly that the green raw citron is an excellent source of vitamin C, the protective dose being very slightly more than 2 g, or approximately 5 to 6 international units per gram based on an estimated protective level of 0.5 mg of ascorbic acid. At the 2-g feeding level the average weight gain per guinea pig was 250 g with only traces of scurvy observed at autopsy. At all higher levels, protection was complete and very large weight gains were recorded for all the animals. The 10-g level of the brined, desalted citron is not shown in figure 2 since the three guinea pigs died within 35 days with an average scurvy score of 9.

The brined, fermented citron showed but a trace of vitamin C, as 15 g gave very little more protection than the basal ration (negative control). That a slight amount of vitamin C was present is indicated by the fact that the guinea pigs receiving 15 g lived 10 to 15 days longer than those receiving only the basal ration. It is safe to conclude from this experiment that the preserved or candied citron which is made from the desalted, brined fruit contains little or no vitamin C. The causes of the loss of vitamin C during fermentation in a 5-percent NaCl brine are not clear. There is much carbon dioxide given off during the fermentation, and since the citrons are under the brine, there is little chance for oxidation. Upon removal from the brine,

the caps were placed directly in boiling water and kept there until they were well desalted. The desalted citron was sliced and fed to the animals as soon as it was cool.

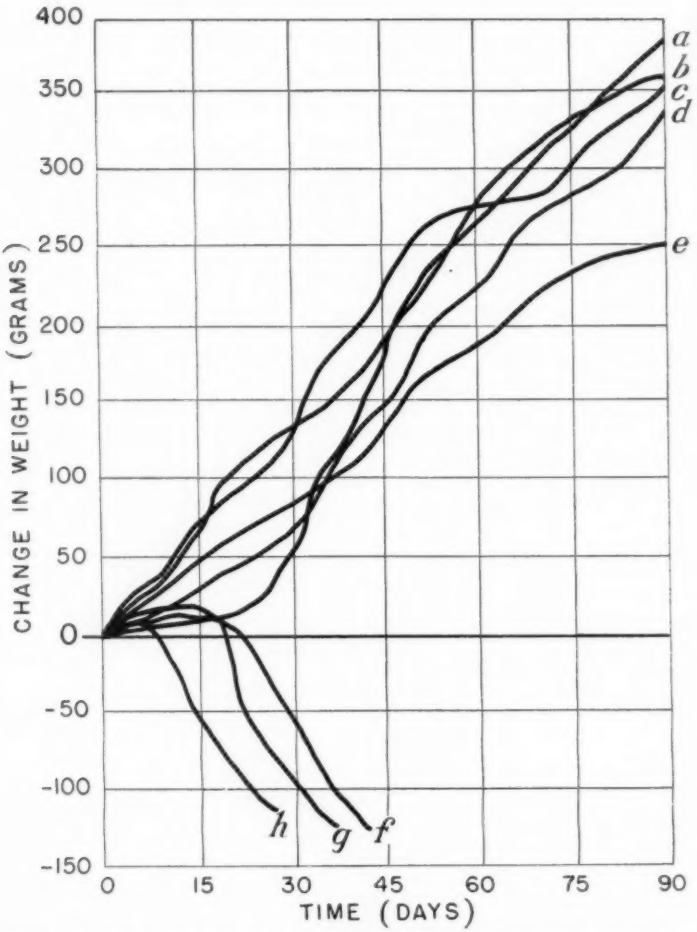

FIGURE 2.—Results of feeding guinea pigs green and brined citron as the sole source of vitamin C: *a*, 12 g green citron fed daily, scurvy score, 0; *b*, 4 g green citron fed daily, scurvy score, 0; *c*, 15 g green citron fed daily, scurvy score, 0; *d*, 6 g green citron fed daily, scurvy score, 0; *e*, 2 g green citron fed daily, scurvy score, trace; *f*, 15 g desalted fermented citron fed daily, scurvy score, 15; *g*, 12 g desalted fermented citron fed daily, scurvy score, 14; *h*, negative control, scurvy score, 19.

The results here reported compare favorably with those of Thurman and Vahlteich (*19*), who found that cucumber pickles manufactured from brined stock gave no measurable protection from scurvy at the 15-g level. Similarly, Eddy and his associates (*6*) showed that cabbage lost about 95 percent of its vitamin C when thoroughly cooked

in an open kettle. Clow, Marlatt, et al. (*4*) and also Parsons and Horn (*14*) found that cabbage suffered marked losses in vitamin C when it was fermented into sauerkraut.

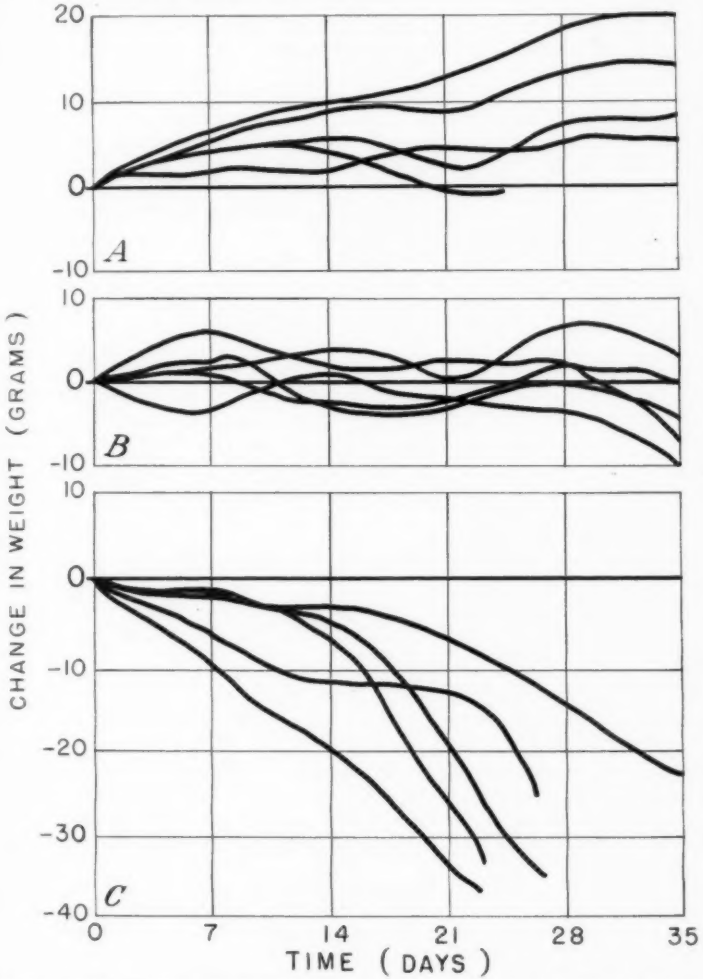

FIGURE 3.—Results of feeding fresh green and preserved citron as the sole source of vitamin A to albino rats previously depleted of this vitamin: *A*, 1 g fresh green citron; *B*, 1 g preserved citron; *C*, negative control receiving vitamin A-free basal ration only.

VITAMIN A IN FRESH GREEN AND PRESERVED CITRON

For determining vitamin A the Sherman and Burtis (*16*) method was used. Young albino rats weighing from 35 to 50 g were fed a vitamin A-free basal diet consisting of cornstarch 67 parts, vitamin-free casein 18 parts, irradiated yeast 10 parts, sodium chloride 1 part,

and Osborne and Mendel's (*13*) salt mixture 4 parts. Normally, the animals are depleted of vitamin A after 4 to 5 weeks on this ration, but in this experiment a somewhat longer period was required before xerophthalmia was observed. Five rats were used at the 1- and 0.25-g levels for both the green fresh citrons and the preserved peel (candied citron), and eight were used for negative controls. Three of the latter died within 2 weeks, and the data for these are not included in the graph for negative controls (fig. 3, C). The sample of preserved peel that was fed contained 19 percent moisture.

The data for the three groups of rats are presented in figure 3. Only the graphs showing weight gains of the rats on the 1-g level are given because of the incomplete protection from xerophthalmia and infections of the sinuses and middle ear of the rats fed 0.25 g. There is some indication that the green fresh citron was somewhat richer in vitamin A than the preserved citron. The latter, however, contains over 50 percent of sugar. In no instance did the citrons give weight gains of 3 g a week, the equivalent of 1 Sherman unit. The vitamin A content of the green fresh citron is estimated roughly at 0.8 to 1.0 international unit, and the preserved citron at slightly less.

SUMMARY

The citron is characterized by high moisture and medium sugar, ash, and fat content. The fiber content is low. Ash analysis shows moderate amounts of calcium, potash, phosphorus, and copper. The iodine content is low.

The fresh unripe citron is very rich in vitamin C, about 2 g being the protective amount for guinea pigs. This corresponds to 6 international units per gram. There is very little retention of vitamin C in the brine-fermented citron and probably none in the candied peel.

The vitamin A content of the green fresh citron is estimated at 0.8 to 1 international unit and that of the preserved (candied) peel at slightly less.

The findings of Hollande and Chadefaux in regard to the microorganisms responsible for the characteristic brine-fermentation of Corsican citron are corroborated for Puerto Rican citron. A yeast, *Saccharomyces citri medicae*, and *Bacillus citri medicae*, were found in large numbers in the fermenting brined citron. The acid production in 15 days, during fermentation, averaged 6 to 7 g per liter (acetic). The flavor and aroma of citron are largely developed during the fermentation.

In preserving citron the use of 35 percent of glucose with the sucrose yields a peel of excellent flavor, texture, and color. A moisture content of preserved (candied) citron peel of 17 to 19 percent is sufficiently low to prevent yeast fermentation or mold growth.

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EFFECT OF FERTILIZER ON QUALITY AND CHEMICAL COMPOSITION OF CANNING PEAS[1]

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INTRODUCTION

Many investigators have studied the chemical composition of fresh peas under various conditions (*1*, *5*, *12*, *13*),[3] and some have studied the composition of canned peas (*26*). Differences in the quality of the peas due to differences in composition have generally only been inferred. Few investigators have considered the effect of plant nutrients on the chemical composition of peas (*11*, *23*), or determined the ultimate effect of plant nutrients or chemical composition on the quality of the canned product as determined by experienced judges (*15*, *23*).

In the present investigation field trials were conducted to determine the influence of fertilizers on the yield and quality of canned peas. Chemical analyses were also made on the fresh whole peas and on pea skins to determine, if possible, the manner in which the changes in quality were brought about.

FIELD METHODS

Two series of plots were laid out in 1931 in the two most important pea-canning areas of Wisconsin. One series was located at Dorchester on Colby silt loam, an old glacial soil derived largely from granitic material, which had a pH value of 5.5 to 6.0. The other series was located at Ripon on Miami silt loam, a soil derived from limestone. The pH value of this soil was 6.6 to 6.8.

Each series of plots included the eight treatments indicated in table 1. The fertilizers were applied broadcast at the rate of 300 pounds per acre and were disked in before planting. The potassium in the fertilizers was derived from muriate of potash, the phosphorus from treble superphosphate, and the nitrogen from ammonium sulphate (70 percent) and from nitrate of soda (30 percent). The Perfection variety was planted on the Colby plots at the rate of 4½ bushels per acre, and the Advancer variety on the Miami plots at the rate of 4 bushels per acre. All the seed was inoculated.

The Miami plots were one-tenth of an acre in size, and the Colby plots were one-fifteenth of an acre. All treatments were duplicated. The Miami plots were seeded on April 22 and harvested on July 3. Hot weather and the danger of increased aphid infestation made it necessary to harvest these plots before the peas had reached the normal canning stage. The Colby plots were seeded May 4. One set of the duplicate plots was harvested on July 13 and the other set on July 15.

[1] Received for publication Apr. 24, 1936; issued January 1937.

[2] The authors acknowledge their indebtedness to Dr. W. E. Tottingham, associate professor of agricultural chemistry, for criticisms offered and for supervision of the sugar and starch analyses.

[3] Reference is made by number (italic) to Literature Cited, p. 878.

The peas from each plot were vined, washed, and graded separately, and each grade (size) from each plot was blanched, canned, and processed separately.[4] The time when each plot was cut, vined, washed, and blanched was recorded. An effort was made to reduce the time between vining and washing to a minimum and to keep this time interval uniform for all plots. Since the viners were located at some distance from the canneries, however, from 1¼ to 3¼ hours elapsed between the time the peas were vined and the time they were washed.

PLOT YIELDS AND QUALITY OF THE CANNED PRODUCT

After the canned peas had been stored for several months, representative cans of each size, from no. 1 through no. 5, were scored by experienced judges. The acre yields and average score of the peas for the various treatments are given in table 1.

TABLE 1.—*Effects of different fertilizer treatments on yield and quality of two varieties of canning peas*

Fertilizer[1] mixture	Perfection variety on Colby plots		Advancer variety on Miami plots	
	Yield per acre	Score[2]	Yield per acre	Score[2]
	Pounds		*Pounds*	
Blank	1,351	84.1	675	80.0
0-16-0	1,294	80.5	678	87.6
0-16-8	1,182	84.2	679	84.0
2-16-4	1,535	86.1	685	93.0
2-16-8	1,622	86.9	712	91.0
2-16-16	1,545	86.2	784	93.0
4-16-16	1,588	84.2	806	88.2
4-16-8	1,518	84.7	636	84.5

[1] Proportions of nitrogen, phosphorus, and potash.
[2] Average of all sizes.

Peas from some of the Colby plots harvested on July 13 were lost, and only the results of the second harvest are given. Rather high temperatures necessitated harvesting the Colby plots somewhat before the peas had reached the normal canning stage; consequently, the yields are slightly below normal. Temperatures above normal and injury from aphids so reduced the yields on the Miami plots that the duplicate plots had to be combined in order to get sufficient material to process each size of pea separately. It had been planned to make two separate harvests on both the Colby and the Miami plots so that if a certain treatment tended to hasten maturity, the peas would be cut at the proper stage of maturity at the early harvest, and if some other treatment tended to delay maturity, the peas would be cut at the proper stage of maturity at the later harvest. Under the conditions prevailing in this investigation these plans could not be carried out.

On the Colby silt loam, those plots that received complete fertilizers produced somewhat higher yields than the unfertilized plots, while the plots that received only phosphorus or phosphorus and potash without nitrogen yielded less than the unfertilized plot. The differences in yield between fertilized and unfertilized plots on the

[4] Made possible torough the generous cooperation of the Libby, McNeill & Libby Co., and the Centra Wisconsin Canneries.

Miami silt loam were not very great because of abnormally high temperatures, aphid infestation, and early harvesting. The highest yields, however, were obtained by the use of complete fertilizers, excepting the 4–16–8 treatment.

The National Canners Association, in their score card for quality of canned peas, give 35 points for tenderness, 25 for flavor, 15 for clearness of liquor, 15 for freedom from broken peas and other defects, and 10 for uniformity in size and color. The last three items are controlled almost entirely by factory practices. Any effect of fertilizer on the quality of canned peas must, therefore, be sought in variations in tenderness and flavor. Canning practices may influence to some extent the flavor and possibly the tenderness of peas, but these factors depend largely on the stage of maturity of the peas and on the variations in chemical composition and cellular structure that accompany variations in maturity.

There were no pronounced differences in the quality of the peas from the Colby plots, but the peas that scored highest were from the plots that received complete fertilizers containing 2 percent of nitrogen, 16 percent of phosphoric acid, and potash varying from 4 to 16 percent. When the nitrogen in the fertilizer was increased to 4 percent, as in the 4–16–8 and 4–16–16 treatments, or left out entirely, as in the 0–16–0 and 0–16–8 treatments, the quality of the peas produced was no better than that of the peas from the unfertilized plot. The 0–16–0 treatment produced the lowest quality peas.

In the Miami silt loam, all of the plots that received fertilizer produced peas of distinctly better quality than the unfertilized plot. As in the Colby silt loam, those plots that received fertilizers containing 2 percent of nitrogen, 16 percent of phosphoric acid, and potash varying from 4 to 16 percent (2–16–4, 2–16–8, and 2–16–16 treatments) produced the highest scoring peas. Increasing the nitrogen to 4 percent, as in the 4–16–8 and 4–16–16 treatments, or leaving the nitrogen out entirely, as in the 0–16–0 and 0–16–8 treatments, resulted in the production of peas that were higher in quality than peas from the unfertilized plot, but lower in quality than the high-scoring peas from the three previously mentioned plots.

The relative differences in quality between treatments, as shown by the average score of all sizes, was in general also exhibited separately by each of the five sizes. For example, the peas from the 2–16–16 plot (Miami silt loam) scored 90, 95, 95, 95, and 90 for the sizes 1 through 5, respectively, giving an average score of 93. The 4–16–16 scored 90, 90, 90, 90, and 81 for the same sizes, giving an average score of 88.2. The peas from the unfertilized plot were the most variable, scoring 86, 76, 81, 81, and 76 for the sizes from 1 through 5, respectively, giving an average score of 80.

The liquor was reported as clear for the cans of peas from all of the plots on the Miami soil. The flavor was reported as being flat for the no. 1 peas from the unfertilized, 0–16–8, and 4–16–8 plots; for all of the other sizes the flavor of the peas from all of the plots was reported as generally good. Tough peas were reported (Miami soil) for all sizes of peas from the unfertilized and the 0–16–8 plots, and for several sizes from the plots receiving the 0–16–0, 4–16–8, and 4–16–16 treatments. Only the no. 1 peas from the 2–16–16 plot were pronounced tough, and the no. 3 peas from the 2–16–8 plot were reported as slightly so. The tenderness of the peas of all sizes from

the 2–16–4 plot was good. From the Miami plots one can thus conclude that the effect of fertilizers on quality of canned peas is due largely to changes in the tenderness of the peas, but the judges' reports indicate that flavor may also be influenced by fertilizers. The peas from the Colby plots did not exhibit such decided differences in tenderness.

The effect of fertilizers, especially of well-balanced complete mixtures, was to improve the culinary qualities of peas grown on the soil types under investigation and to increase the yield of peas. From the results reported some general conclusions may be drawn as to the approximate composition of a fertilizer that will improve the quality as well as increase the yield of the pea crop. When applied at the rate of 300 pounds per acre, such a fertilizer (for Wisconsin conditions on the Colby and Miami silt loams) should contain about 2 percent of nitrogen, 16 percent of phosphoric acid, and from 4 to 16 percent of potash.

CHEMICAL COMPOSITION OF PEA SKINS

Peas for the seed-coat analyses were withdrawn from the plot lots after they had gone through the washer and graders. The seed coats were removed by hand as soon as possible. About 5 g of the fresh skins was preserved for total nitrogen determination, and about 50 g for calcium, potassium, and phosphorus. Accepted methods of quantitative analysis were followed in determining the amounts of mineral elements.

TOTAL NITROGEN

The standard Kjeldahl procedure was followed in determining the total nitrogen content of the pea skins. The results are given in table 2.

TABLE 2.—*Effect of different fertilizer treatments on the total nitrogen content (dry basis) of skins from no. 2, no. 3, and no. 4 peas grown on the Colby plots*

Fertilizer mixture	Nitrogen in skins of—			Fertilizer mixture	Nitrogen in skins of—		
	No. 2 peas	No. 3 peas	No. 4 peas		No. 2 peas	No. 3 peas	No. 4 peas
	Percent	*Percent*	*Percent*		*Percent*	*Percent*	*Percent*
Blank	4.79	3.70	3.35	2–16–16	4.04	3.82	3.51
0–16–0	4.18	3.86	3.47	4–16–8	4.37	3.82	3.38
0–16–8	4.46	4.14	3.55	4–16–16	4.13	3.64	3.38
2–16–8	4.40	3.62	3.43				

[1] See footnote 1, table 1.

Marked differences are found in the nitrogen content of pea skins of different sizes. All fertilizers apparently lowered the nitrogen content of the no. 2 peas, but with the larger sized peas this effect is not consistent. The general trend, irrespective of treatment, shows a consistent decrease in the nitrogen of the seed coat of peas with advancing maturity, assuming that the larger peas are more mature. This fact indicates that the total nitrogen content of pea skins is primarily a function of maturity and not one of nitrogen added in the form of commercial fertilizer.

If the assumption is correct that larger peas are more mature (*19*, *23*), then the smaller percentage of nitrogen in the larger peas may be explained by the fact, established by Bisson and Jones (*1*), that total nitrogen increases less rapidly than carbohydrates and other nonnitrogenous constituents. Thus it appears that the total nitrogen in the skins is influenced by the maturity factor in a way similar to the whole pea.

TABLE 3.—*Effect of different fertilizer treatments on the calcium, phosphorus, and potassium content of skins from peas grown on the Colby plots*

Fertilizer mixture	Pea size no.	Calcium on—		Phosphorus on—		Potassium on—	
		Ash basis	Dry matter basis	Ash basis	Dry matter basis	Ash basis	Dry matter basis
		Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Blank	2	3.79	0.151	11.29	0.450	32.60	1.30
	3	4.39	.178	10.17	.414	38.06	1.55
	4	5.21	.208	8.33	.333	33.24	1.33
0-16-0	2	3.40	.147	12.48	.541	32.44	1.41
	3	3.96	.167	11.25	.475	32.56	1.38
	4	4.39	.190	9.93	.480	32.47	1.41
0-16-8	2						
	3	3.45	.175	11.03	.496	30.65	1.38
	4	4.06	.176	11.02	.478	31.88	1.38
2-16-8	2	3.46	.145	12.08	.507	32.47	1.36
	3	4.07	.173	10.36	.441	33.58	1.43
	4	5.20	.217	8.97	.375	31.42	1.32
2-16-16	2	3.52	.165	13.38	.627	31.77	1.49
	3	3.90	.170	11.47	.496	33.33	1.44
	4	4.73	.200	9.23	.390	30.70	1.30
4-16-8	2						
	3	4.12	.169	11.19	.468	28.07	1.15
	4	4.78	.204	10.60	.452	29.21	1.25
4-16-16	2	3.70	.157	13.55	.574	29.49	1.34
	3	3.97	.172	11.38	.497	31.92	1.38
	4	4.87	.205	8.83	.393	32.13	1.36

[1] See footnote 1, table 1.

PHOSPHORUS

Table 3 gives the percentage of phosphorus, potassium, and calcium in the pea skins. The phosphorus content of the various fertilizer mixtures was uniformly 16 percent. In practically every case, the phosphorus in pea skins decreases regularly with increase in size of pea, following in general the nitrogen trend. Phosphorus accumulates in relatively large amounts in the ovules in the earlier stages of growth and this appears also to be true of the seed coats. Rather striking increases in phosphorus in the seed coats are noted. These increases are consistent and particularly marked in the no. 2 and no. 4 peas, amounting to 25 and 28 percent, respectively (average of all fertilizer treatments).

POTASSIUM

No such definite relationships are noted in potassium as in nitrogen and phosphorus. Nor did the inclusion of various amounts of potassium in the fertilizer exert any consistent influence on the potassium content of the seed coats. This is particularly noticeable if such treatments as 0–16–0 and 0–16–8 or 2–16–8 and 2–16–16 are compared. The skins of no. 3 peas from the unfertilized plot contained a larger percentage of potassium than the skins of no. 3 peas from any of the fertilized plots. The peas on the unfertilized plot apparently were able to obtain as much potassium as those on plots receiving potash

fertilizers. It is possible that the potassium content of the rather inert skin tissues is quite constant and does not vary greatly even though more potassium may be present in the vegetative part of the plant (*23*).

The potassium comprised from 28 to 38 percent of the ash of pea skins. A similar high concentration of potassium in pea skins has been reported by others (*23*). From 8.3 to 13.5 percent of the ash of pea skins was phosphorus, and from 3.4 to 5.2 percent was calcium. It is not known what significance, if any, this high percentage of potassium in the ash of pea skins has.

CALCIUM

It will be remembered that tenderness is given more weight than any other single factor in scoring canned peas for quality. Tough seed coats are associated with a lack of tenderness in peas, although it must not be overlooked that the character of the pulp may also influence tenderness. This is true whether mechanical means (*23*) are used to determine tenderness or whether it is determined by experienced judges.

The results of numerous investigations indicate a relationship between calcium and potassium in plant tissue. Fonder (*7*) reports that in alfalfa a high potassium content is usually accompanied by a low calcium content, and, inversely, a low potassium content is accompanied by a high calcium content. A similar relationship has been reported between calcium and potassium in peas (*23*, *25*). Sayre, Willaman, and Kertesz (*23*) found that calcium in pea ovules decreases as the potassium increases, and that a higher calcium content is always associated with tougher peas.

In the present investigation, a relationship was observed between the calcium content of the pea skins and potash in the fertilizers. A comparison of the seed coats of peas from the 0–16–0 and the 0–16–8 plots shows that the addition of potash reduced the calcium in the seed coats of the no. 3 and no. 4 peas. A comparison of the seed coats of peas from the 2–16–8 and 2–16–16 treatments gave similar results, except in the case of no. 2 peas. The differences between the effect of the 4–16–8 and the 4–16–16 treatments are negligible. In general, potash in the fertilizer served to diminish the concentration of calcium in the seed coats of peas, a finding which is in harmony with those reported by others (*23*, *25*).

It is apparent that maturation also influences calcium concentration in the seed coat. Without exception, a definite increase in the calcium of the seed coats is noted with an increase in size of peas. On the plots receiving potash the average increase in calcium of the seed coats of no. 3 over no. 2 peas is about 10 percent and of no. 4 over no. 3 about 20 percent. A similar condition is found in the unfertilized plot, but the magnitude of the increase is even greater in the no. 3 as compared with the no. 2 peas. Since calcium migration to the skin is an old-age pnenomenon, it is very probably associated with the deposition of old-age organic constituents, such as hemicelluloses, lignins, or pectates. In the case of whole peas the calcium content appears to be fairly constant irrespective of the stage of maturity (*5*, *23*, *26*).

It is a striking fact that all of the fertilizer treatments reduced the calcium concentration in the seed coats, whether phosphorus alone,

phosphorus with potash, or all three elements were provided. It is possible that larger yield and more active metabolism diminished the proportion of calcium migrating to the skin and also the ratio of skin to total seed. It is to be noted, however, in comparing the various fertilizer treatments, that there is no definite relationship between the relative percentages of calcium found in the pea skins and the quality of the canned product. For example, the peas from the 2–16–8 treatment received the highest score, but the pea skins from this plot had a higher calcium content than the pea skins from most of the other fertilized plots. Also, the lowest concentration of calcium was in the seed coats of peas from the plot receiving the 0–16–8 treatment. This treatment did not give the highest scoring peas.

Calcium probably occurs in plant tissues not in the inorganic state but combined or associated with organic constituents such as lignins, pectates, and hemicelluloses. These organic constituents may have a direct influence on the quality of peas by affecting the cellular structure and thus the tenderness and flavor of the peas. Calcium associated with these organic constituents may thus be but an indirect indication of quality in peas.

CHEMICAL COMPOSITION OF WHOLE PEAS

The starch content of whole peas may vary from less than 6 to over 37 percent and the total sugars from above 35 to less than 4 percent (*1*, *5*, *23*). The relative amounts of these two constituents in peas have always been considered important in affecting quality.

Since stage of maturity is of prime importance in determining sugar and starch content, and thus the flavor and tenderness of peas, many workers have investigated the organic constituents of peas at various stages of growth. Boswell (*2*, *3*, *4*, *5*) states that the growth and maturity process is characterized by a rapid decrease in percentage of sugar and soluble nitrogenous substances, and by an increase in starch, total and acid-hydrolyzable substances, and insoluble nitrogen compounds. Sayre and his coworkers (*23*) report a decrease in sugar and an increase in total organic matter and protein as the pea ovule matures. Bisson and Jones (*1*), in an investigation covering 5 weeks' development, during which time 14 samplings were made, found that peas contained a maximum of 33.5 percent of sucrose, but that before the peas were at the proper stage for harvesting the sucrose content had dropped to 20.5 percent. They found that the total sugar in peas per pod continued to increase slowly while the percentage was decreasing until the proper stage for harvesting was reached, when the total sugar per pod and the percentage both decreased. The ratio of starch to sugar has been used by various investigators (*2*, *5*, *19*) to correlate what was considered the most important chemical changes in the development of the pea with maturity and quality.

Sufficient evidence is thus at hand to show that the quality of peas changes with stage of maturity and that this change in quality is accompanied by a decrease in sugars and an increase in starch. However, it has not been determined whether nutrient elements supplied in fertilizers can affect the organic composition of the pea and thus the quality. Jodidi and Boswell (*11*), working with an abnormal soil and using heavy applications ot nitrogen, phosphorus, and potash singly, concluded on the basis of 2 years' work that the application of

nitrogen to peas (116 pounds of nitrogen per acre) resulted in a higher percentage of sucrose and a distinctly lower starch content. The phosphorus treatment (160 pounds P_2O_5 per acre) was followed by no noticeable difference in percentage of carbohydrate constituents, ash, or of the ether extract. One hundred and forty-four pounds of potash per acre apparently had no effect on the sugar or starch content. Sayre's work (*22*, *23*) shows quite conclusively that the mineral composition of peas and of pea skins is affected by the nutrients supplied and that high calcium in the seed coat is associated with a tougher and harder pea, which results in a lower quality canned product. Sayre found that the structure of the cells of the pea ovule was definitely affected by differences in the nutrients supplied. However, he concluded (from water-culture experiments) that the pea plant can vary its mineral content and the ratios among these minerals over a very wide range and yet not appreciably alter its ratio of carbohydrates and nitrogenous constituents. This may be an unsafe generalization, since desiccation and maturation of the pea ovule may be disturbed and delayed by the constant high plane of water in water cultures.

TABLE 4.—*Effect of different fertilizer treatments on the sugar and starch content starch-sugar ratios, and alcohol-soluble matter of no. 3 peas grown on the Colby and the Miami plots*

Fertilizer mixture[1]	Total dry matter soluble in 70-percent alcohol		Starch				Sugar				Starch-sugar ratio	
			Miami		Colby		Miami		Colby			
	Miami	Colby	Ratio to total dry matter	Total dry matter insoluble in 70-percent alcohol	Ratio to total dry matter	Total dry matter insoluble in 70-percent alcohol	Ratio to total dry matter	Total dry matter soluble in 70-percent alcohol	Ratio to total dry matter	Total dry matter soluble in 70-percent alcohol	Miami	Colby
	Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*		
Blank	40.0	49.2	12.98	21.6	9.65	19.0	20.17	50.3	29.36	59.5	0.643	0.328
0–16–0	43.8	48.5	11.06	19.7	9.66	18.7	22.41	51.1	28.59	58.8	.493	.337
0–16–8	43.6	52.4	11.62	20.6	7.72	16.2	22.18	50.8	26.81	51.1	.524	.288
2–16–8	39.9	41.9	11.91	19.8	11.50	19.7	21.41	53.6	31.80	75.7	.556	.361
2–16–16	41.7	48.2	11.62	19.9	9.92	19.2	24.05	57.5	29.09	60.2	.483	.341
4–16–8	36.4	50.7	13.05	20.5	8.68	17.6	18.40	50.5	.....	.....	.709	.....
4–16–16	.....	46.9	.....	.....	9.91	18.6	.....	.....	20.88	44.1	.....	.474

[1] See footnote 1, table 1.

Table 4 gives the results of sugar and starch analyses made on whole peas in connection with the fertilizer trials on quality. The peas for analysis were taken at the canning factory after they had gone through the washer and grader. They were preserved in 70 percent alcohol ($CaCO_3$ was also added to some), and the analyses were made according to official methods.[5] Sugar content represents sucrose, since reducing sugars were not present.

It would be expected that the peas having the highest sugar and the lowest starch content, and thus the lowest starch-sugar ratio, would give the highest quality peas when canned. There is, however, no consistent relationship between sugars and starch content and the

[5] Bertrand's method of reducing the copper in Fehling's solution, and Mohr's volumetric method of determining the cuprous oxide, were followed.

effect of fertilizer on quality. Moreover, it should be noted that while the Perfection peas from the Colby silt loam were considerably higher in sugar and lower in starch than the Advancer peas from the Miami soil, the latter produced a higher quality canned product.

It has already been mentioned that the peas were cut before they reached the normal canning stage. Most canners consider a pea crop at the proper canning stage when 24 to 32 percent of the crop consists of no. 1, no. 2, and no. 3 peas. The peas on the Miami plots averaged 57 percent and on the Colby plots 34 percent of no. 1, no. 2, and no. 3 peas. When peas are at this stage of maturity and are still high in sugars and low in starch other constituents may be more important in determining the quality of the canned product than the sugar and starch content.

The starch and sugar together comprised about 30 to 40 percent of the total dry matter of the peas. While these two constituents undoubtedly are important in determining quality, the importance of the various nitrogenous constituents and carbohydrates other than sucrose and starch has been stressed by numerous investigators. Boswell (*5*) suggested the use of an insoluble-soluble nitrogen ratio but did not use it because its effect on quality is not so well known. Attempts have been made in Germany (*17*), France (*8, 19, 20, 21*), and the United States (*15*) to detect adulterated peas by means of various ratios of organic constituents other than starch and sugars. While it is important that all these constituents should be considered in any analysis for quality, no one has yet been able to set any definite ratio of any constituents as a critical or deciding point between superior and inferior quality peas.

Numerous investigators (*6, 10, 12, 13, 14, 15, 18*) have reported on the changes in composition of peas after harvesting. Peas were kept for from several hours to a week at different temperatures and chemical changes noted. Fresh green peas which were kept for as long as a week at 0° C. showed no appreciable change in chemical composition. Sugar was lost rapidly if the peas were held at high temperatures, even if only for a few hours. Most writers have shown, or attempted to show, that with a decrease in sugar there is a concomitant increase in starch. If the data are examined carefully, the increase in starch and other polysaccharides is found never to equal the loss in sugar. Most of the sugar is lost in respiration, and the increase in percentage of starch, as Kertesz (*14*) has shown, is due to the fact that the same amount of starch after loss of sugar by respiration must give a higher percentage. In another investigation (*15*) where peas were held for varying periods of time, Kertesz and Green were not able to correlate observed changes in quality of the canned product with changes in organic constituents.

The loss of sugars may be the primary cause of deterioration in the quality of market peas, but this can hardly be the explanation of the deterioration in canned peas since sugar is added in considerable quantity to the brine used in canning. This addition of sugar does not make the flavor of low-grade peas equal to that of choice peas.

SUMMARY

The influence of various fertilizers on the yield and quality of canning peas was studied in the field in 1931 on the Colby and Miami silt loams of Wisconsin. Properly balanced fertilizers increased the

yield of peas and also improved the quality of the canned peas, as determined by experienced judges.

Calcium, potassium, phosphorus, and total nitrogen determinations were made on the seed coats of no. 2, no. 3, and no. 4 peas from the Colby fertilizer plots. All of the fertilizers decreased the calcium and increased the phosphorus content of the seed coats of the three sizes of peas studied. The variations in calcium content of the seed coats bore no definite relation to quality of the canned product. The nitrogen and potassium content of the pea skins was not consistently affected by the fertilizer treatments. The skins of larger peas consistently contained more calcium and less phosphorus and total nitrogen than those of the smaller peas.

Sugar and starch analyses of no. 3 peas from the various fertilizer plots on both the Colby and Miami soils were made. Variations in quality of the canned peas could not be explained by differences in the sugar and starch content of the fresh peas. The peas on all plots were harvested at a relatively early stage of maturity, with the result that the peas were relatively high in sugar and low in starch. It is probable that variations in quality of canned peas harvested at such relatively early stages of maturity are determined largely by variations in organic constituents other than sugar and starch.

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